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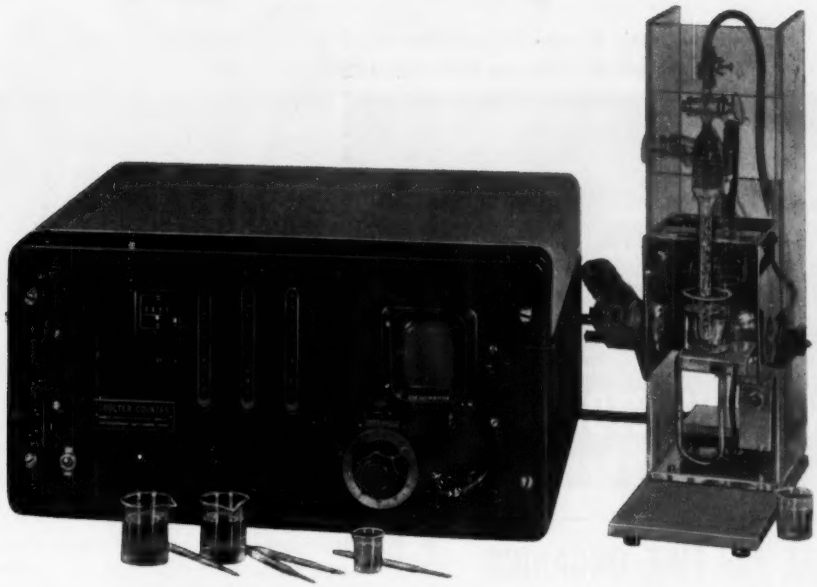

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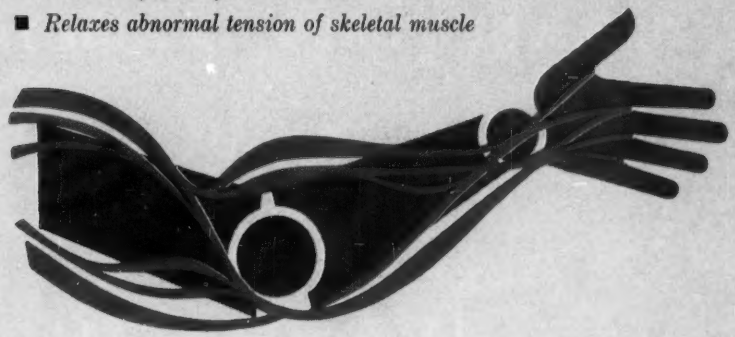

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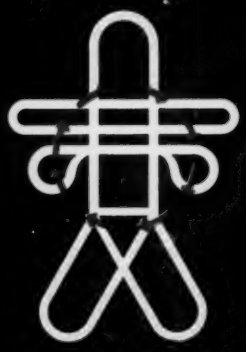

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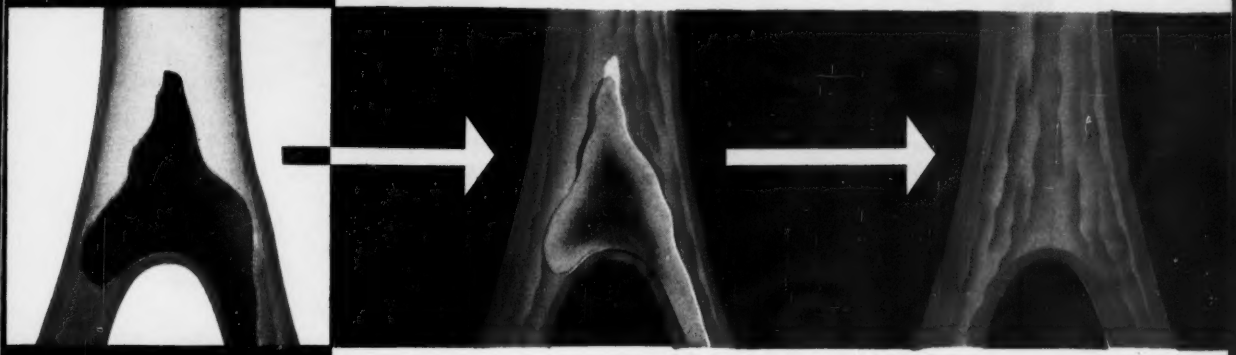

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WATER AND ELECTROLYTE STUDIES IN CHOLERA *

By RAYMOND H. WATTEN,† FRANCIS M. MORGAN,† YACHAI NA SONGKHLA,‡ BUNAM VANIKIATI ‡ AND ROBERT A. PHILLIPS †

(From the United States Naval Medical Research Unit No. 2 (NAMRU-2), Taipei, Taiwan (Formosa), and the Department of Medicine, Chulalongkorn University School of Medicine, Bangkok, Thailand)

(Submitted for publication March 17, 1959; accepted June 19, 1959)

Rational treatment of cholera cannot be provided without precise knowledge of the water and electrolyte losses which occur in this disease; such information is not available in the literature. Studies prior to 1900 usually reported electrolyte concentrations in whole blood, not in plasma, and sodium and chloride were frequently reported as NaCl. Since 1900 there have been many reports of electrolyte concentrations in plasma or fecal excreta but only one preliminary report (1) relates fecal losses to plasma levels in the same patient. However, this report suffers, as do all others on cholera, from the fact that oral intake was not restricted and it is not certain to what extent fecal electrolytes had been diluted with oral fluids.

The 1958 cholera epidemic in Bangkok, Thailand, provided the opportunity to perform quantitative, volumetric studies of water and electrolytes in plasma, feces and urine for the first 24 hours after admission in patients suffering from cholera. During this period oral food and fluids were withheld and the dehydration and acidosis were treated by intravenous fluids. The effect of water and sodium loading on electrolyte exchange was also observed and whole blood and plasma analyses were repeated on each patient in convalescence.

MATERIALS

Twenty-five patients with diarrhea were admitted to a study ward provided by the Chulalongkorn University Medical School at the Bangkok Red Cross Society Hospital during June and July, 1958. A positive culture of *Vibrio comma* was obtained on 17 of these patients once or more often. As the 1958 epidemic was confined principally to Bangkok and nearby suburbs, patients were able to reach well-staffed hospitals soon after the onset of their symptoms. The majority of patients were received in fair condition; however, several patients were in moderate shock with systolic blood pressures under 100 mm. Hg. There was one death: an 83 year old woman with electrocardiographic evidence of cardiac disease died three days after admission.

A venous blood sample was obtained on admission; thereafter blood, urine, and feces were collected at intervals of one to four hours during the first 24 hours, then less frequently until the diarrhea ceased. Convalescent blood samples were taken prior to discharge. Five patients left against advice soon after their diarrhea ceased; some of these "convalescent" values may not accurately represent their normal blood composition.

Care was taken to avoid spillage of feces. A large bore rectal tube was used for the first 24 hours in addition to a cholera bed in which the patients' buttocks were placed over an opening in the bed frame and excreta collected in five gallon containers. Retention catheters were placed in the bladder for the first 24 hours to facilitate urine collection. Urine and feces were measured in graduated cylinders to the nearest 5 ml. Aliquots of fecal material were filtered through glass wool and a Seitz filter. Blood samples were heparinized and the plasma separated immediately after a sample for duplicate hematocrits and whole blood specific gravities had been obtained. Fecal specimens and blood for plasma CO_2 content were collected under oil and analyzed within 20 minutes.

During the first 24 hours of observation and treatment, patients were given nothing by mouth with the exception of four who were given carmine dye with 100 ml. of water in order to measure bowel transit time. After 24 hours, fluids were given *ad libitum* and a diet of rice gruel, boiled eggs and milk was allowed. All patients received an antibiotic, either chloramphenicol or a tetracycline derivative, after the 24 hour observation period and had at least one cholera-negative stool culture prior to discharge. Patients 13 and 18 probably had cholera but repeated attempts to culture the *V. comma* were unsuccessful.

* The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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METHODS

The following procedures were performed: whole blood (Gb) and plasma (Gp) specific gravities (2); microhematocrit (Vc) (3); carbon dioxide content of plasma and feces (4); sodium and potassium concentrations of plasma, feces and urine by internal standard flame photometry; and a Fiske osmometer was used for osmolarity determinations of plasma and feces. All determinations were done in duplicate and the results averaged.

The intravenous treatment schedule recommended by Johnson, Weaver and Phillips (5) was followed using the copper sulfate method (2) for determination of Gp. Normal saline was infused rapidly in amounts calculated to correct the initial dehydration (200 ml. for each 0.001 increase in Gp above 1.025). Following this, fluids were given to match measured urine and fecal losses or to correct elevations in Gp. A detailed evaluation and discussion of this method based on the results of the present study will be presented elsewhere (6).

Sodium bicarbonate was infused as a 2 or 4 per cent solution to treat acidosis and potassium was given in small amounts. In order to obtain more information on electrolyte exchange, ideal treatment schedules were not adhered to in all patients. Several of the later patients were given nonelectrolyte (5 per cent dextrose in distilled water) and hypertonic (2 per cent saline) or Roger's solution (1.35 per cent saline) infusions in an attempt to alter fecal electrolyte concentrations. The epidemic subsided before additional planned experiments could be completed.

RESULTS

Table I lists pertinent admission data and 24 hour intake and output volumes for the 17 proven cholera patients. For comparison the table has been divided into two sections: patients having over 3 L. of fecal excretion and patients having under 3 L. during the first 24 hours.

Tables II and III list the results of plasma and whole blood examinations performed at the following times: immediately on admission, following initial rehydration, 24 hours after admission and in convalescence. All plasma electrolytes, CO_2 contents and osmolarity values have been cor-

Table I

Twenty-four hour intake and output and fecal electrolyte concentrations in cholera patients with (A) over 3 liters fecal excreta and (B) under 3 liters

Patient	Sex	Age	Weight Kg	I. V. fluids Volume	Urine Volume	Fecal Volume	Fecal electrolyte contents Na	Cl	K	CO_2	Osmolarity
							m Eq / L				mOsm/L
					A						
1	M	28	65	10, 200	1, 425	3, 465	130	68	33. 3		337
2	M	83	45	18, 100	305	11, 430	165	107	9. 5		328
3	M	30	43	12, 100	1, 795	6, 750	142	109	15. 6		291
5	F	15	36	6, 700	1, 005	3, 125	152	123	13. 6		307
6	F	30	35	19, 400	210	7, 700	135	130	14. 8		311
8	M	38	55	13, 250	360	8, 320	139	107	16. 5		324
10	F	23	34	10, 700	1, 650	5, 650	119	104	16. 7	46. 3	259
12	F	23	40	10, 500	1, 480	7, 260	132	113	14. 3		297
14	M	58	58	13, 000	855	10, 000	129	93	16. 0	51. 4	279
15	M	34	55	21, 000	1, 175	17, 435	142	118	9. 7	42. 7	279
21§	M	23	52	12, 880	1, 665	8, 240	122	100	17. 3	40. 2	282
Mean				13, 200	1, 085	8, 125	137	107	16. 1	45. 2	300
					B						
7*	F	83	30	9, 220	930	2, 885	139	121	16. 2		288
9	F	46	42	9, 600	1, 690	2, 870	131	105	24. 9		273
20§	M	57	37	11, 200	2, 630	2, 700	123	114	27. 8	31. 7	285
23§	M	22	52	6, 000	1, 335	880	82	58	40. 0	60. 5	232
24§	M	23	39	4, 700	3, 030	1, 870	122	78	23. 9	45. 9	266
25§	F	35	50	3, 500	545	2, 860	85	60	25. 7	35. 7	254
Mean				7, 370	1, 700	2, 345	114	89	26. 4	43. 5	266

* Died

§ Treated with non-electrolyte intravenous solutions as well as with normal saline

TABLE II

Whole blood and plasma values in patients with over 3 liters fecal excreta per 24 hours

Patient	Period	Time	Vc	Gb	Gp	CO_2*	Na*	Cl*	K*	Osmolarity*	Total
						mEq/L				mOsm/L	Protein§
	Admission		62	1.071	1.040	13.7	168	118	6.5		11.9
	Rehydration	2 hrs.	48	1.058	1.028	14.0	168	127	6.3		7.8
1	24 Hours		38	1.052	1.021	24.0	152	118	3.0	373	5.2
	Convalescence	2 days	38	1.051	1.021	22.1	152	120	3.8		5.2
	Admission		52	1.067	1.042	22.2	162	119	4.5		13.0
	Rehydration	2 hrs.	50	1.065	1.032	18.3	149	116	4.3		9.3
2	24 Hours		31	1.045	1.023	27.6	160	122	2.8	364	5.9
	Convalescence	7 days	35	1.050	1.023	29.3	157	120	3.8	324	5.9
	Admission		55	1.068	1.038	19.4	168		4.8	424	11.2
	Rehydration	1/2 hr.	42	1.051	1.024	18.4	153	119	4.4	345	6.3
3	24 Hours			1.052	1.024	15.2	152	124	4.0	335	6.3
	Convalescence	3 days	39	1.052	1.026	22.8	154	117	3.9		7.0
	Admission		47	1.059	1.033	19.5	151	118	3.8	326	9.6
	Rehydration	1 hr.	33	1.043	1.024	18.0	155	131	3.4	288	6.3
5	24 Hours		33	1.047	1.025	17.0	149	130	3.5	321	6.7
	Convalescence	3 days	35	1.050	1.025	26.5	155	121	3.3	314	6.7
	Admission		44	1.060	1.038	15.0	149	111	3.5	337	11.2
	Rehydration	2 hrs.	29	1.044	1.026	16.3	152	126	3.1	323	7.0
6	24 Hours		25	1.043	1.025	14.9	162	146	2.8	350	6.7
	Convalescence	4 days	25	1.040	1.022	28.0	149	112	2.2	296	5.5
	Admission		61	1.073	1.043	22.1	158	113	5.2		13.4
	Rehydration	1-1/2 hr.	44	1.055	1.030	21.1	150	124	4.6	336	8.5
8	24 Hours		41	1.052	1.027	23.1	159	124	3.9	359	7.4
	Convalescence	4 days	41	1.053	1.028	27.4	142	108	3.3	301	7.8
	Admission		57	1.068	1.041	14.0	157	129	4.8	364	12.7
	Rehydration	1 hr.	39	1.050	1.025	17.0	156	137	6.8	347	6.7
10	24 Hours		31	1.047	1.024	23.1	166	131	3.3	327	6.3
	Convalescence	6 days	32	1.047	1.025	37.0	153	112	3.3	341	6.7
	Admission		50	1.064	1.041	11.1	155	129	4.3	377	12.7
	Rehydration	1-1/2 hr.	41	1.054	1.031	15.4	155	135	6.2	380	8.9
12	24 Hours		29	1.046	1.025	18.5	169	139	2.9	353	6.7
	Convalescence	3 days	32	1.048	1.025	30.4	151	116	3.1	295	6.7
	Admission		51	1.060	1.032	22.7	149	118	4.5	337	9.3
	Rehydration	1 hr.	42	1.054	1.029	22.1	148	124	4.9	367	8.1
14	24 Hours		41	1.053	1.026	23.9	152	120	3.1	321	7.0
	Convalescence	6 days	36	1.052	1.026	28.1	148	112	3.3	334	7.0
	Admission		62	1.072	1.039	20.5	155	117	4.9		11.5
	Rehydration	1/2 hr.	52	1.059	1.029	23.0	147	124	6.4	391	8.1
15	24 Hours		38	1.054	1.026	40.3	177	128	2.8	373	7.0
	Convalescence	7 days	43	1.052	1.023	33.3	152	110	3.4		5.9
	Admission		59	1.070	1.040	19.2	155	116	5.9		12.3
	Rehydration	2 hrs.	48	1.058	1.029	19.4	145	119	6.3	338	8.1
21	24 Hours		37	1.051	1.025	25.9	156	125	3.6	332	6.7
	Convalescence	2 days	42	1.053	1.028	22.8	147	119	4.0	316	7.8
	Mean Values										
	Admission		54.4	1.067	1.039	18.1	158	119	4.8	361	11.7
	Rehydration		42.6	1.054	1.028	18.5	153	126	5.2	346	7.7
	24 Hours		34.4	1.049	1.025	23.0	159	128	3.2	346	6.5
	Convalescence		36.2	1.050	1.025	28.0	151	115	3.4	315	6.6
	Normal Ranges		40-44	1.056-1.058	1.025-1.027	25.7-30.0	146-156	107-114	3.8-5.4	300-322	7.2-7.8

Vc = hematocrit; Gb = blood specific gravity; Gp = plasma specific gravity.
* Converted to mEq or mOsm per liter plasma water (see text)
§ Derived from Gp (8).

rected for plasma protein concentrations by conversion to mEq. or mOsm. per L. of plasma water using Eisenman, MacKenzie and Peters' formula (7) Wp = 98.4 − (0.718Pp) where Wp = plasma water and Pp = plasma protein concentration.

On admission the extent of dehydration is evident from the elevation of Gp and of plasma osmolarity. Vc and Gb are higher than convalescent values but in several instances (Patients 6, 7 and 25) they are in the range of normal due to severe

TABLE III

Whole blood and plasma values in patients with under 3 liters fecal excreta per 24 hours

Patient	Period	Time	Vc	Gb	Gp	CO_2*	Na*	Cl*	K*	Osmolarity*	Total Protein§
							m Eq / L				
	Admission		40	1.057	1.029	19.7	151	113	4.6	339	8.1
	Rehydration	2 hrs.	30	1.048	1.028	18.3	150	121	4.0	349	7.8
7	24 Hours		31	1.049	1.025	17.4	151	126	3.6	360	6.7
	Convalescence	(Died)									
	Admission		54	1.064	1.034	21.5	152	122	5.2	343	10.0
	Rehydration	2-1/2 hrs.	41	1.055	1.025	17.6	153	137	5.1		6.7
9	24 Hours		39	1.049	1.023		156	139	3.2	338	5.9
	Convalescence	4 days	37	1.050	1.022	23.8	150	116	3.3	303	5.5
	Admission		57	1.064	1.034	17.0	151	121	7.5	337	10.0
	Rehydration	3 hrs.	47	1.058	1.027	15.6	154	128	5.8	329	7.4
20	24 Hours		36	1.047	1.023	22.6	153	126	5.7	325	5.9
	Convalescence	2 days	38	1.049	1.023	35.5	145	119	4.2	323	5.9
	Admission		53	1.065	1.035	19.8	167	117	5.4		10.4
	Rehydration	1 hr.	47	1.058	1.030	21.1	137	108	4.0	347	8.5
23+	24 Hours		37	1.049	1.025	31.7	154	115	3.8	322	6.7
	Convalescence	2 days	38	1.051	1.026	28.2	151	116	3.5	322	7.0
	Admission		53	1.062	1.032	23.4	148	112	2.8		9.3
	Rehydration	1 hr.	46	1.054	1.026	22.9	139	104	3.2		7.0
24+	24 Hours		42	1.052	1.026	24.1	143	118	3.9	323	7.0
	Convalescence	4 days	41	1.049	1.024		136	116	3.5	290	6.3
	Admission		41	1.059	1.034	13.2	151	124	4.2	327	10.0
	Rehydration	2 hrs.	30	1.048	1.027	21.5	154	127	3.4	319	7.4
25	24 Hours		28	1.046	1.025	19.3	147	122	3.2		6.7
	Convalescence	2 days	29	1.048	1.028	20.0	148	121	3.5	316	7.8
	Mean Values										
	Admission		49.7	1.062	1.033	19.1	153	118	5.0	337	9.6
	Rehydration		40.2	1.054	1.027	19.5	148	121	4.3	336	7.5
	24 Hours		35.5	1.049	1.025	23.0	151	124	3.9	334	6.5
	Convalescence		36.6	1.049	1.025	26.9	146	118	3.6	311	6.5
	Normal Ranges		40-44	1.056-1.058	1.025-1.027	25.7-30.0	146-156	107-114	3.8-5.4	300-322	7.2-7.8

Vc = hematocrit; Gb = blood specific gravity; Gp = plasma specific gravity.
* Converted to mEq or mOsm per liter plasma water (see text)
§ Derived from Gp (8).
\+ Initially rehydrated with 5 per cent dextrose in distilled water.

anemia. When electrolyte and osmolarity determinations are corrected for the volume occupied by plasma proteins, mean sodium and chloride levels in patients with over 3 L. of fecal excreta are higher than those considered to be normal and are also higher than the patients' own convalescent values. In addition to dehydration, acidosis is evident in all patients.

Following the period of initial rehydration which took from 30 to 180 minutes and involved infusions of 1.5 to 3.6 L. of normal saline, marked improvement in hydration as measured by Gp and osmolarity occurred. There was no appreciable change in plasma CO_2 content as bicarbonate solutions were not given during this period. Elevated chloride values seen on admission persisted during the treatment.

Observations at the end of 24 hours of treatment indicated improvement in CO_2 content and maintenance of fluid balance. It will be noted that in most patients values for plasma sodium and chloride are elevated above values found in convalescence.

Vc and Gb values in convalescence revealed severe anemia in several patients. Hypoproteinemia was apparent in several patients but was not as severe nor as frequent as anemia. These low values were probably due to the poor nutritional state and to parasitic infections of the socio-economic class who were cholera victims. A mild alkalosis persisting into convalescence was observed in Patients 10, 12, 15 and 20.

Table I lists individual volumes, electrolyte concentrations, osmolarities and their mean values in

TABLE IV

Fecal volumes and electrolyte concentrations during the first 24 hours in patients with non-cholera diarrhea

Patient	Volume	Na	Cl	K	CO_2	Osmolarity
			m Eq / L			mOsm/L
11	480	45.1	41.8	29.4		310
18	870	71.0	29.0	29.1	47.0	300
22	290	113.0	59.6	10.7	48.9	297
Mean	545	76.4	43.5	23.0	47.9	302

the feces of cholera patients. Of interest are the high concentrations of potassium and carbon dioxide in the excreta. Data are available (Table IV) on fecal electrolyte concentrations in three patients with diarrhea not caused by cholera. Other non-cholera patients did not have sufficient watery stools during the observation period.

Figure 1 relates plasma and fecal concentrations of sodium and intravenous intake and fecal output volumes during treatment. A close correlation between plasma and fecal sodium levels is apparent. The patient, an 83 year old man, was treated cautiously as his renal function and cardiac reserve were unknown. Acidosis was effectively

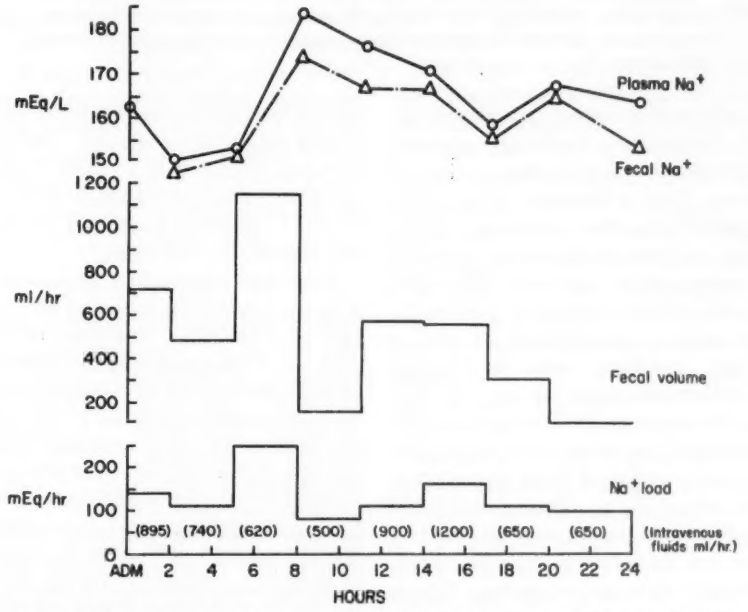

FIG. 1. PATIENT NO. 2. PLASMA AND FECAL SODIUM CONCENTRATIONS, FECAL VOLUME AND VOLUME OF INTRAVENOUS FLUIDS DURING VARYING SODIUM LOADS

Isotonic sodium chloride was infused continuously at different hourly rates. Between hours five and eight, 4 Gm. $NaHCO_3$ per 100 ml. was added to the infusion.

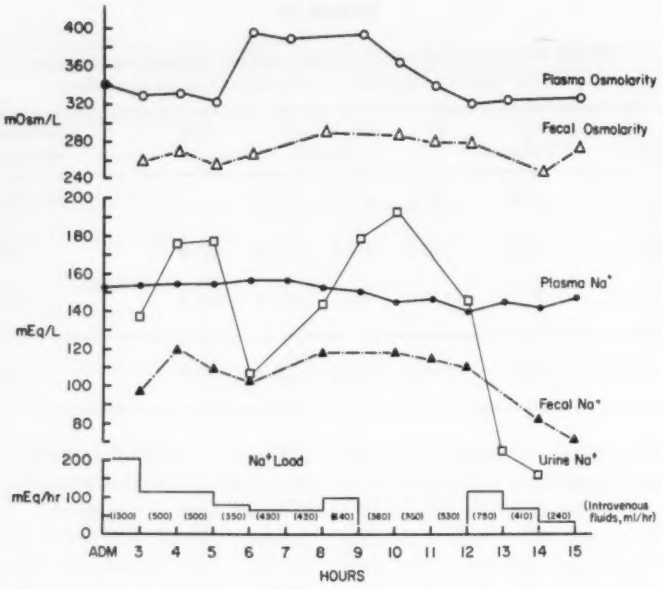

FIG. 2. PATIENT NO. 20; RESPONSE OF PLASMA, FECAL AND URINARY SODIUM AND PLASMA AND FECAL OSMOLARITY TO SODIUM AND WATER LOADING

Between hours 9 and 12, 5 per cent dextrose in water was substituted for normal saline. Following water loading fecal sodium concentration decreases. Proportionate increase in fecal potassium levels (not shown) maintains fecal osmolarity.

corrected but rehydration, based on observed fecal and urine volumes, was inadequate probably because the patient sequestered a large volume of feces in his bowel from a temporary ileus which was released several hours after admission. When this was realized, intravenous fluids were increased and adequate urine output was soon established.

Figures 2 and 3 show changes in plasma, fecal and urine sodium concentrations as well as plasma and fecal osmolarity values in response to periods of sodium and water loading. In Figure 2 the most obvious change in plasma and fecal sodium concentrations occurs after water loading induced by the infusion of 5 per cent glucose in water at the rate of 30 ml. per minute for 180 minutes. There is a sharp drop in plasma sodium after one hour but the decrease in fecal sodium does not occur for an additional two hours. Plasma and fecal osmolarities parallel these changes. Observed mouth to anus transit time with carmine dye in this patient was three and one-half hours. In Figure 3 there are less striking changes in plasma and fecal sodium levels but the values show a close degree of correlation. Plasma osmolarity increased sharply with a sodium load of 265 mEq. per hour; fecal osmolarities showed similar increases. Observed mouth to anus transit time in this patient was two hours.

From data obtained on 10 patients it was possible to calculate the extent of plasma CO_2 elevation after infusions of sodium bicarbonate solution. The relationship of the amount of sodium bicarbonate infused to increase in plasma CO_2 content is shown in Figure 4. It was determined that 0.46 to 0.8 mEq. of sodium bicarbonate per Kg. of body weight (mean 0.58) would raise the plasma CO_2 content 1 mEq. per L. These values are not corrected for fecal loss of HCO_3^- during the infusion period and indicate approximate sodium bicarbonate requirements for treating the acidosis of cholera patients who had an active diarrhea. Urinary HCO_3^- losses were assumed to be negligible because postinfusion elevation of plasma HCO_3^- did not exceed the normal range. There

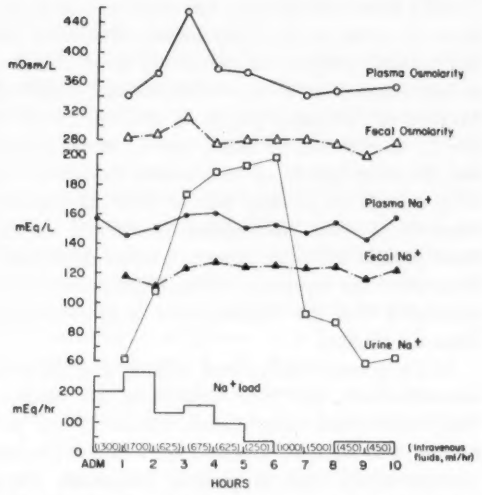

FIG. 3. PATIENT NO. 21; RESPONSE OF PLASMA, FECAL AND URINARY SODIUM AND PLASMA AND FECAL OSMOLARITY TO WATER LOADING

Between hours six and eight, 5 per cent dextrose in water was substituted for normal saline.

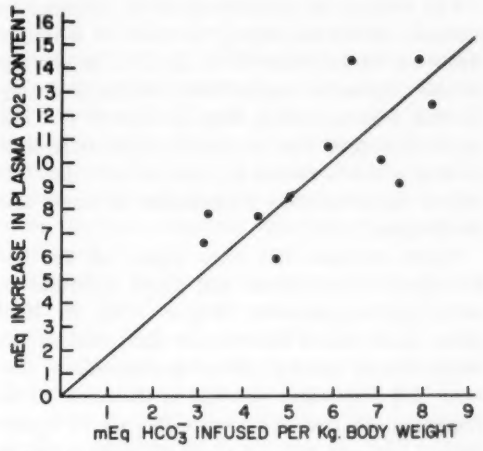

FIG. 4. RELATIONSHIP BETWEEN SODIUM BICARBONATE INFUSED AND RESULTING ELEVATION OF PLASMA CO_2 CONTENT

The line represents a plot of the mean value (0.58 mEq. per Kg.).

is evidence that urinary excretion of HCO_3^- is insignificant when plasma CO_2 levels are under 25 mEq. per L. (9). It was possible to correct for the fecal loss of HCO_3^- in several patients where fecal volume and fecal HCO_3^- concentration were known. Again, urinary loss of HCO_3^- was assumed to be negligible. In these patients it was found that a mean value of 0.41 mEq. per Kg. of infused HCO_3^- as sodium bicarbonate caused an increase of plasma CO_2 of 1 mEq. per L.

DISCUSSION

The necessity for fluid and electrolyte replacement in acute cholera is obvious. However, published literature provides no unanimity of opinion on how much fluid and how much electrolyte should be given because balance-type studies relating fecal to plasma concentrations in patients deprived of oral intake and given only intravenous fluids have not been reported heretofore. Referring to Table I, 24 hour fecal volumes averaged over 8 L. in the more severe cases. Total stool volumes during the two or three days of diarrhea averaged over 13 L. Although several 24 hour urine volumes are low, these were due to maintenance of minimal hydration; no patient in this series had evidence of ischemic renal damage. Urine output was found to be a reliable index of hydration because adequate renal function was established following infusions sufficient to lower Gp below 1.027.

The degree of dehydration in cholera patients cannot be estimated with accuracy by clinical signs but must be determined by measurement of hemoconcentration. Hemoglobin concentration and hematocrit have been found unreliable because cholera patients are not infrequently anemic (5). Admission electrolyte determinations are also of no assistance in assessing the severity of dehydration. Plasma sodium concentrations were usually higher in patients who were more severely dehydrated on admission but no correlation between plasma sodium levels and Gp was apparent in patients with Gp under 1.035. Comparison of Tables II and III also indicates no correlation between concentration of other electrolytes and the varying degrees of dehydration observed.

Plasma protein concentration was found to be a reliable guide to the extent of dehydration because convalescent plasma protein concentrations varied less from patient to patient than hemoglobin or hematocrit. The copper sulfate method (2) for determining plasma proteins was used during this study because of its accuracy, simplicity and adaptability to field conditions.

The volume of intravenous fluids required to maintain hydration during the first 24 hours of treatment varied from 3.5 to 21 L. The average patient required a total of 16 L. of fluids during his entire illness with a range of 6 to 35 L. The rapid prolonged loss of fluid of high electrolyte content which persisted for two to three days required the continuous replacement of water and electrolytes.

Much emphasis has been placed on the observation of low serum and blood chlorides in acute cholera patients. Rogers (10) in 1909 found lower blood chlorides in fatal cholera patients than in healthy Indians and postulated that more salt than water was lost in the feces. This reasoning led him to advocate the use of hypertonic (1.35 per cent) sodium chloride solutions which would he stated, "maintain the conservative beneficial hypertonicity of the blood, which would tend to carry more fluid into the circulation instead of removing it through the damaged bowel wall." The reporting of low plasma electrolyte concentrations by Rogers and by others is to be expected, since they reported their results as concentrations per volume of plasma or serum without correcting for the large amounts of plasma proteins in the blood of severely dehydrated cholera patients. In this study, several patients had low plasma chloride concentrations before correction for plasma protein content. While plasma measurements here have been corrected for protein concentration, such correction has been ignored in the reporting of electrolytes and osmolarity in the feces (which were all Seitz filtered) as the concentration of protein in Seitz filtered cholera stools is negligible, averaging from 0.1 Gm. per 100 ml. (Weaver, Johnson and Phillips) (1) to 0.2 Gm. per 100 ml. (Saha and Das) (11).

As long ago as 1849, Becquerel (12) postulated that the feces in cholera were a transudate that poured out through the damaged bowel wall. Nearly all writers on cholera who have studied the nature and composition of cholera stools have adopted this view. The most complete recent study of untreated cholera patients has been reported by Saha and Das (11) who injected sodium thiocyanate into patients with acute cholera and were able to detect this material in every sample of stool examined one to two hours after the injection. They also administered the blue dye T-1824 to several patients but were unable to recover it in the stool. They include studies of the biochemical composition of cholera feces obtained at admission prior to any treatment and found an average of 125 mEq. per L. of sodium, 76 mEq. per L. of chloride, 19 mEq. per L. of potassium and 31 mEq. per L. of bicarbonate (measured as CO_2 content). In only two of their 23 patients were all of these electrolytes determined on the same patient and simultaneous plasma determinations were not reported. From their studies they concluded that the cholera stool is a transudate from the plasma.

In the present study, fecal sodium and chloride concentrations, and fecal osmolarity, are consistently lower than their plasma values. Fecal potassium and HCO_3^-, however, are found in greater concentrations than in plasma, potassium being two- to sixfold and HCO_3^- nearly double the plasma levels. It is evident (Tables I and IV) that as 24 hour stool volumes increase, potassium concentrations decrease and the lowest values are found in patients with the most severe diarrhea. Fecal bicarbonate concentrations do not appear to be as closely related to fecal volume as the other ions. High concentrations of potassium (11, 13–15) and bicarbonate (11, 14) in diarrheal stools have been reported previously and are not unique to cholera.

In the adult, fecal excreta normally contain some potassium but almost no sodium and chloride (Elkinton and Danowski) (16) due to absorption of these ions in the lower ileum and colon. With the onset of diarrhea the concentrations of sodium and chloride in the feces increase in proportion to the fecal volume and approach plasma levels when the volume of excreta is over 3 L. per 24 hours (Tables I and IV). Increasingly rapid intestinal transit time as a result of larger stool volumes probably contributed to these elevated values by allowing insufficient time for reabsorption, but the extent of this contribution is unknown. Mouth to anus transit time with orally administered carmine dye was 90 and 150 minutes in two patients with over 8 L. of fecal excreta per 24 hours and 225 and 240 minutes in two cases with 24 hour fecal volumes of under 3 L. These observations indicate that transit time is shortened as fecal volume is increased and that sodium and

chloride conservation in the intestinal tract is related inversely to transit time.

Further evidence of the existence and variability of gastrointestinal potassium losses in abnormal conditions is supplied by the observations of Bernstein and colleagues (17) who produced diarrhea in uremic patients by perfusing the entire gastrointestinal tract with electrolyte solutions. Elevation of potassium levels in the intestinal perfusate in excess of the starting concentration was evident in most of their patients. In addition, the elevation of potassium in the intestinal perfusate was greater at a flow of 1 L. per hour than it was at 3 L. per hour.

Experimental studies in recent years have altered classical concepts of electrolyte movement and exchange in the mammalian gastrointestinal tract. In acute and chronic experiments in dogs, D'Agostino, Leadbetter and Schwartz (18) prepared colonic pouches into which they instilled isotonic saline. Specimens taken at frequent intervals during the following eight hours demonstrated that as sodium and chloride concentrations fell, potassium and HCO_3^- increased. Potassium concentrations reached an average of 15 to 20 mEq. per L. within three to four hours with little if any further increase. Bicarbonate concentrations increased steadily for the first four to five hours levelling off at CO_2 content of 50 to 70 mMoles per L. Comparable results were obtained when experiments were repeated after the colonic pouch had been treated with antibiotics. Visscher and co-workers (19, 20), using a sodium isotope and deuterium in dogs, have shown that this ion and water move in both directions across the intestinal epithelium even against large concentration gradients. They estimated that the total turnover rate of sodium was quite large, being approximately equal to the total plasma sodium every 83 minutes.

The origin of the copious diarrhea in cholera is not known. Nor is there any information regarding the site and extent of water and electrolyte exchange between the extracellular fluid and the gut lumen in this disease. It remains to be determined if the diarrhea fluid in cholera is the result of: *1*) a transudative process; *2*) an increase of normal gastrointestinal secretions; *3*) failure of normal water and electrolyte reabsorption; or *4*) a combination of these abnormalities. The transudate theory has been used for many years to explain the large fecal volumes in cholera. Reports of autopsy findings reviewed by Pollitzer (21), in which desquamation of the intestinal mucosa has been described, lends support to this concept. It is difficult, however, to reconcile the rapid return of bowel function, in patients recovering from cholera, with widespread mucosal damage; hence other diarrhea-provoking mechanisms should be considered.

Alteration of the reabsorptive capacity of the bowel by the *V. comma* could explain the source of the large fecal volumes if the transfer of water and electrolytes into the gut is not impaired. Using Visscher's figure of 83 minutes for clearance of sodium from the plasma, and assuming plasma volume to be 5 per cent of body weight, then, in a 55 Kg. man, the bowel would receive about 48 L. of isotonic fluid in 24 hours. Patient 15 (Table I) had a fecal volume in excess of 17 L. in 24 hours and, if this mechanism applies, it would indicate a 35 per cent impairment in the efficiency of his reabsorptive mechanism. In cholera it is quite obvious that reabsorption of fluids from the gut lumen is not normal and while transit time is rapid it is doubtful that decreased transit time is solely responsible for the diarrhea. On the other hand, if reabsorption were to be inhibited to varying degrees by the *V. comma* or its products, Visscher's studies provide a simple mechanism to explain the origin of the diarrhea fluid.

A close relationship between plasma and fecal electrolyte concentrations is evident in Figure 1. The same relationship exists in Figures 2 and 3 but of a smaller magnitude and with a lag in fecal concentrations presumably due to slower evacuation of the fecal volume. The renal response to both water and salt loading in preserving plasma sodium levels is demonstrated in Figures 2 and 3. It is also evident that while water and salt loading cause corresponding changes in fecal electrolytes, the bowel does not approach the kidney in ability for selective excretion of water and electrolytes.

Quantitative requirements for the treatment of acidosis were studied by infusing varying amounts of sodium bicarbonate into several cholera patients and observing changes in the plasma CO_2 content. Palmer and Van Slyke (22), reported that a 70 Kg. man would require 1.8 Gm. of oral sodium bicarbonate to elevate the plasma CO_2

concentration one volume per cent (0.69 mEq. per Kg. for 1 mEq. increase in HCO_3^-). Hence they assumed HCO_3^- to be distributed equally in total body water. In this study the infusion of 0.58 mEq. of sodium bicarbonate per Kg. body weight was required to increase plasma CO_2 content 1 mEq. (Figure 4). If corrections are made for the amount of HCO_3^- lost in the feces during the infusion period, a mean value of 0.41 mEq. per Kg. is found. This is identical with the value found following rapid infusion (100 ml. per minute) of a 4 per cent bicarbonate solution into patients without diarrhea during convalescence from typhoid fever (23).

In the present cholera study, bicarbonate was not given until the patients had been rehydrated. In these patients, assuming that extracellular water is 20 per cent and cellular water is 40 per cent of total body weight and that HCO_3^- is distributed equally throughout extracellular water, then for each mEq. increase of HCO_3^- in extracellular water, total cellular water will show an increase of only 0.5 mEq. The observation that the infusion of 0.41 mEq. of HCO_3^- per Kg. body weight will elevate plasma HCO_3^- by 1 mEq. is in accord with more recent observations in the literature. Pitts has reported (9) that the intravenous infusion of 15.7 mMoles of sodium bicarbonate per Kg. body weight into a nephrectomized dog will result in plasma HCO_3^- of 33.8 mEq. per L. or 0.46 mEq. per Kg. for 1 mEq. elevation per L. of plasma.

Singer and associates (24) infused hypertonic (0.89 molar) sodium bicarbonate into 12 normal subjects. Calculations from their data presented in Figure 3 indicate that the infusion of 0.46 mEq. per Kg. body weight resulted in the elevation of plasma HCO_3^- by 1 mEq. per L. However, in their discussion they stated that 0.35 mEq. per Kg. was a more representative value for their experiments. The present studies do not provide information as to the distribution of HCO_3^- ions in body fluids. This has been carefully studied and discussed by Singer and associates (24) and by Pitts (9). The present studies provide, rather, additional information on the quantity of sodium bicarbonate required to treat acidosis in patients with and without diarrhea.

The loss of large amounts of electrolyte in the feces of cholera patients indicates the necessity for adequate replacement. In addition to the sodium and chloride loss it is evident from Table I that for each liter of feces lost from cholera patients, an average of 45 mEq. of HCO_3^- and 16 mEq. of potassium should be given. From the elevated sodium concentrations and osmolarity values observed on admission and during treatment with isotonic saline (Tables I and II) evidence is provided that treatment with hypertonic solutions is not necessary and may be harmful, particularly in the presence of impaired renal function or low cardiac reserve. There is also sufficient evidence to condemn the use of nonelectrolyte solutions as the chief replacement fluid in cholera. Two patients (23 and 24, Table III) were initially rehydrated with 5 per cent dextrose in water. Low plasma sodium levels were observed at the end of the rehydration period and Patient 24 developed severe muscular cramps which were not relieved until 200 ml. of a 2 per cent solution of NaCl had been infused.

For the first time detailed, quantitative information has been provided on the water and electrolyte losses in cholera. However, the extent of water and electrolyte shifts between cellular and extracellular spaces cannot be determined from our data; this must await further study on cholera victims.

SUMMARY

1. Twenty-five patients with diarrhea were studied during the 1958 Bangkok cholera epidemic; 17 were proven to have had cholera. For the first 24 hours after admission patients were given nothing by mouth. During this period the infusion of intravenous electrolyte solutions was related to hydration and electrolyte concentrations in plasma, feces and urine.

2. Hyperosmolarity and elevation of plasma sodium and chloride were found on admission. Fecal osmolarity and fecal sodium and chloride concentrations were lower than corresponding plasma values while fecal potassium and bicarbonate levels were considerably higher.

3. The relationship between fecal electrolyte concentrations, volume and transit time is presented, and the significance of these findings in relation to experimental studies on active transport in bowel is discussed.

4. In correcting the acidosis of cholera the infusion of 0.58 mEq. of HCO_3^- as sodium bicarbo-

nate effected an elevation of plasma CO_2 of 1 mEq. per L. When correction for the fecal loss of HCO_3^- during the infusion period was made, this value was found to be 0.41 mEq. per Kg.

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THE RENAL EXCRETION OF URIC ACID IN PATIENTS WITH GOUT AND IN NONGOUTY SUBJECTS * †

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The elevation of the plasma uric acid concentration in patients with gout has been attributed by various authors to: *a*) decreased destruction of uric acid, *b*) overproduction of uric acid, or *c*) an abnormality in the renal excretion of uric acid (1). Decreased destruction of uric acid in patients with gout has not been found in studies utilizing labeled uric acid (2). A metabolic abnormality in the production of uric acid has been demonstrated in some patients with gout; however, increased production of uric acid has not been a uniform finding (3, 4).

The possibility that there might be a specific abnormality in the renal excretion of uric acid by patients with gout has been investigated on numerous occasions. The most extensive recent investigations of the renal excretion of uric acid were carried out by Coombs and co-workers (5), Talbott (6) and Gutman and Yü (1), all of whom agreed that there is no essential difference in the manner in which uric acid is excreted by subjects with gout and by normal subjects. However, their studies were performed while the normal subjects and gouty patients had markedly different plasma uric acid concentrations. In the present investigation of the renal excretion of uric acid, patients with gout were studied under control conditions and nongouty subjects were studied before and after they had been given uric acid or its precursors in amounts great enough to elevate their plasma uric acid concentrations to values comparable to those of the gouty patients.

METHODS

Six male patients with gout were studied. One patient was studied on two occasions. Their clinical status is indicated in Table I. In every case, a typical history of acute gouty arthritis was obtained. Gouty patients with renal failure, heart disease or advanced age were purposely excluded from the study. Ten studies were performed on seven nongouty male subjects. The clinical information on this group is also given in Table I.

The control studies were carried out while all of the gouty patients and nongouty subjects were taking their usual diets. They were instructed, however, to eat no high purine foods and to take no uricosuric drugs for at least one month before the studies. The test studies were done after the nongouty subjects had been given uric acid or one of its precursors in addition to their diet. The nature of these dietary supplements is indicated in Table I. Two nongouty subjects were studied after they had been fed a high purine diet. The high purine diet was isocaloric with the control diet and contained the same proportions of fat, carbohydrate and protein, but sardines, liver and anchovies were used to provide 90 per cent of the protein. All of the remaining subjects were given ribose nucleic acid packed in gelatin capsules and administered in four equal doses with meals and at bedtime. One normal subject was also given uric acid and desoxyribose nucleic acid. With one exception, each subject received the dietary supplement for a three day period. On the morning of the fourth day, three hours before the first clearance period, the subjects were given their usual morning dose of the dietary supplements. The two subjects on the high purine diet, however, received no food before the renal function studies on the fourth day. Subject 7 was given ribose nucleic acid for five days and after his usual morning dose of ribose nucleic acid the renal function studies were repeated on the sixth day.

All the studies were started between 7:00 and 8:00 a.m. and completed between 10:00 a.m. and 12:00 noon. The subjects were hydrated by the oral administration of 600 to 800 ml. of water in the first hour and 150 to 250 ml. of water every half hour during the remainder of the study. Standard clearance methods were used (7). Catheterization of the bladder, however, was done only in those cases in which the estimated residual bladder urine exceeded 20 ml. (8). The urine collection periods varied from 10 to 20 minutes in the catheterized patients and 15 to 30 minutes in the voiding patients. All clearances in this report represent the average of the values from two to four sequential clearance periods. Two to four plasma specimens were analyzed during each study. The individual inulin clearances did not vary more than 10 per

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TABLE I

Clinical data and dietary regimens

Patients	Age	SA*	Regimen†	Catheterized‡	Family history	Tophi	Arthritis
	yrs.	*M.²*					*yrs.*
			Nongouty subjects				
1	40	1.76	High purine	0	0	0	0
2	26	1.70	High purine	0	0	0	0
3A	47	1.75	4 Gm. RNA	0	0	0	0
3B			4 Gm. RNA	0	0	0	
4A	37	1.85	4 Gm. RNA	0	0	0	0
4B			4 Gm. DNA	0			
4C			4 Gm. UA	0			
5	23	1.92	4 Gm. RNA	0	0	0	0
6	51	1.74	4 Gm. RNA	0	0	0	0
7	23	1.85	7 Gm. RNA	0	0	0	0
			Patients with gout				
8	55	2.0	0	0	0	0	10
9	52	1.73	0	+	+	0	5
10	49	1.82	0	+	+	0	10
11	44	1.85	0	0	+	+	1
12	28	2.1	0	0	0	0	4
13A	51	2.2	0	+	0	0	6
13B			0	+			

* SA = surface area.
† RNA = ribose nucleic acid, DNA = desoxyribose nucleic acid, UA = uric acid.
‡ A "+" sign in this column indicates that the individual was catheterized during the renal function study.

cent from the mean of the particular study with four exceptions. In these four studies, the uric acid to inulin clearance ratios for the individual periods did not vary more than 5 per cent from the mean of the particular study.

Blood specimens were mixed with heparin and the plasma was separated by centrifugation. The enzymatic [1] spectrophotometric method of Praetorius was used for the determination of uric acid (9). The plasma uric acid determinations reported here were performed directly on the specimens without prior protein precipitation. In addition, enzymatic spectrophotometric uric acid determinations were done on protein-free filtrates of plasma prepared by the method of Forsham, Thorn, Prunty and Hills (10). None of the urine specimens contained protein by the heat and acetic acid test. The uric acid determinations were carried out directly on diluted urine specimens. Inulin was determined by the method of Young and Raisz (11). The anthrone precipitated when used in the concentrations suggested by these authors. Their method worked satisfactorily, however, with 3.2 Gm. of anthrone in place of the recommended 4 Gm. per L. of acid solution and 1.25 ml. of unknown solution in place of the recommended 2 ml. of unknown per 10 ml. of anthrone solution (12). Twenty-four hour urine collections were obtained during the control period and on the last day of each oral loading period. The method of Phillips (13) was used for urine creatinine determinations.

[1] The uricase was obtained through Worthington Biochemical Corporation, Freehold, N. J.

RESULTS

The results of the renal function studies (Table II) have been corrected to a body surface area of 1.73 M.². No correction was made for the results of the 24 hour urine collections. One nongouty subject had an average inulin clearance of 82 ml. per minute. The history, physical examination, urinalysis and intravenous pyelogram on this subject were all within normal limits. One additional nongouty subject and four of the gouty patients had inulin clearances below 100 ml. per minute. In no case, however, was the inulin clearance outside the range of variation for the age or more than two standard deviations below the mean of the subjects studied by Davies and Shock (14).

The mean plasma uric acid concentration was 5.6 mg. per 100 ml. in the nongouty subjects and 9.2 mg. per 100 ml. in the gouty patients under control conditions. Two of the nongouty subjects had plasma uric acid concentrations of 7.1 and 7.2 mg. per 100 ml. In our laboratory, using fresh whole plasma, uric acid concentrations of this level are occasionally seen in normal subjects. These values exceed what is usually considered

TABLE II

*A. Renal function studies on nongouty subjects**

Subjects	P_{UA} C	P_{UA} L	C_{IN} C	C_{IN} L	C_{UA} C	C_{UA} L	F_{UA} C	F_{UA} L	T_{UA} C	T_{UA} L	C_{UA}/C_{IN} C	C_{UA}/C_{IN} L	$U_{UA}V$ C	$U_{UA}V$ L	24 hr. Ur UA C	24 hr. Ur UA L	24 hr. Ur UA/CR C	24 hr. Ur UA/CR L
	mg./100 ml.		*ml./min.*		*ml./min.*		*mg./min.*		*mg./min.*				*mg./min.*		*mg.*			
1	7.1	8.1	80	84	7.7	10.3	5.7	6.8	5.2	6.0	0.096	0.124	0.54	0.83	521	990	0.348	0.642
2	7.2	8.3	104	109	7.6	9.8	7.5	9.1	7.0	8.3	0.073	0.090	0.55	0.82	710	960	0.432	
3_A	4.9	6.8	117	120	9.2	13.8	5.7	8.2	5.3	7.3	0.079	0.115	0.45	0.94	440	520	0.349	0.622
3_B	4.3	6.3	133	136	10.5	14.8	5.7	8.5	5.2	7.6	0.079	0.109	0.45	0.93	442	752	0.243	0.445
4_A	6.0	8.7	124	124	7.8	11.0	7.5	10.8	7.0	9.8	0.063	0.089	0.47	0.96	612	1,400	0.290	0.686
4_B	5.9	7.1	128	139	8.2	11.2	7.6	9.9	7.1	9.1	0.064	0.081	0.48	0.80	490	1,180	0.305	0.607
5	4.7	7.3	106	109	9.0	13.5	5.0	8.0	4.6	7.0	0.085	0.124	0.42	0.98	450	980	0.366	0.622
6	4.5	7.9	95	97	9.5	9.2	4.3	7.6	3.9	6.9	0.100	0.095	0.43	0.73	492	732	0.406	0.564
7	5.5	10.9	107	104	6.7	11.9	5.9	11.4	5.5	10.1	0.063	0.113	0.37	1.31	582	820	0.319	0.354
Mean	5.6	7.9	110	114	8.5	11.7	6.1	8.9	5.6	8.0	0.078	0.104	0.46	0.92	527	926	0.342	0.576
S.D.	1.1	1.3	17	18	1.2	1.9	1.2	1.5	1.1	1.4	0.014	0.016	0.06	0.17	91	260	0.06	0.11

B. Renal function studies on patients with gout

Patients	P_{UA}	C_{IN}	C_{UA}	F_{UA}	T_{UA}	C_{UA}/C_{IN}	$U_{UA}V$	24 hr. Ur UA	24 hr. Ur UA/CR
	mg./100 ml.	*ml./min.*	*ml./min.*	*mg./min.*	*mg./min.*		*mg./min.*	*mg.*	
8	8.9	101	6.6	9.0	8.4	0.066	0.58	1,090	0.614
9	8.9	116	7.4	10.3	9.6	0.064	0.66	465	0.570
10	8.3	85	4.8	7.0	6.6	0.056	0.40	492	0.324
11	9.2	95	6.2	8.7	8.1	0.065	0.57		
12	7.8	92	6.3	7.1	6.6	0.066	0.49	615	0.372
13_A	11.3	98	6.0	11.1	10.4	0.061	0.68	601	0.322
13_B	9.8	112	6.0	10.9	10.3	0.053	0.59	617	
Mean	9.2	100	6.2	9.2	8.6	0.062	0.57	647	0.44
S.D.	1.1	11	0.8	1.7	1.6	0.005	0.10	227	0.14

* The results of the studies under control conditions are listed in columns under "C." Results of studies after loading are listed under "L." The differences of the means of the nongouty subjects after purine loading and the means of the gouty patients were compared by using the "t" test for small series. The probability that these differences occurred by chance alone is less than 0.01 for C_{UA}, C_{UA}/C_{IN} and $U_{UA}V$, and 0.05 for 24 hr. Ur UA. P_{UA} = plasma uric acid, C_{IN} = inulin clearance, F_{UA} = filtered uric acid load, T_{UA} = reabsorbed uric acid, $U_{UA}V$ = urine uric acid during clearance study, 24 hr. Ur UA = urine uric acid in 24 hour collection and UA/CR = mg. of uric acid per mg. of creatinine in 24 hour urine collection.

the upper limits of the normal range of uric acid in protein-free serum filtrates (1). Other investigators have also noted the slightly higher uric acid values obtained with enzymatic spectrophotometric methods on whole plasma (15).

The mean uric acid clearance and proportion of filtered uric acid excreted by the patients with gout was slightly lower and the mean filtered, reabsorbed and excreted uric acid was slightly higher than in the nongouty subjects (Table II and Figure 1). There was some overlap of individual values between the two groups of subjects in every measured parameter with the exception of the plasma uric acid concentration. The mean 24 hour urine uric acid excretion was higher in the patients with gout than in the nongouty group. One patient with gout excreted 1,090 mg. of uric acid per day. All of the other patients with gout for whom we have data excreted between 465 and 617 mg. of uric acid per day, which is approximately the same range as that of the nongouty subjects. In view of the difficulty of collecting 24 hour urine specimens on nonhospitalized subjects, uric acid to creatinine ratios were determined. These were similar in the two groups under control conditions. That the majority of subjects with gout do not excrete an abnormally large amount of uric acid each day in their urine is shown by our data and in the extensive investigations reported by Gutman and Yü (1).

Daily administration of 4 Gm. of uric acid by mouth had little effect on the plasma uric acid concentration or the 24 hour urine urate excretion.[2] The high purine diet and 4 Gm. per day of desoxyribose nucleic acid were moderately effective while 4 Gm. per day of ribose nucleic acid usually resulted in a striking rise in plasma uric acid concentration and in 24 hour urine uric acid excretion. The greatest increase in plasma uric acid concentration occurred in the subject who was given 7 Gm. of ribose nucleic acid per day for

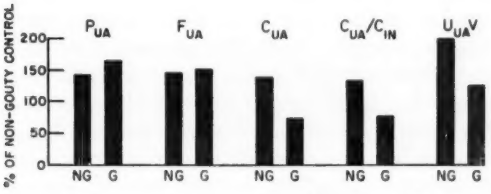

FIG. 1. RENAL FUNCTION OF NONGOUTY SUBJECTS (NG) AFTER ORAL LOADING WITH PURINES COMPARED WITH THAT OF PATIENTS WITH GOUT (G) UNDER CONTROL CONDITIONS

The height of each column represents the mean results expressed as per cent of the nongouty control values. P_{UA} = plasma uric acid concentration, F_{UA} = filtered uric acid load, C_{UA} = uric acid clearance, C_{IN} = inulin clearance and $U_{UA}V$ = rate of uric acid excretion.

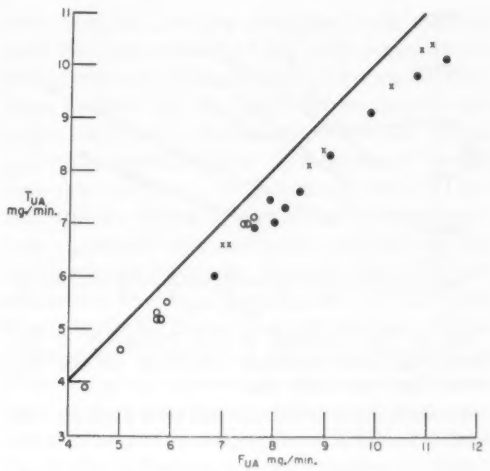

FIG. 2. THE RELATION OF TUBULAR REABSORPTION OF URIC ACID (T_{UA}) TO FILTERED URIC ACID LOAD (F_{UA})

The heavy diagonal line indicates total reabsorption of the filtered load of uric acid. The results of the studies on the nongouty subjects under control conditions are indicated by open circles and after uric acid loading by solid circles. The values in the patients with gout studied under control conditions are indicated by cross marks.

[2] In Study 4_C, after 4 Gm. of uric acid a day for three days, the plasma uric acid concentration was 6.4 mg. per 100 ml. and the filtered uric acid 7.9 mg. per minute. These values differed little from the plasma uric acid concentration and filtered uric acid load of the control studies and were far below the levels of the patients with gout. Therefore, the results of the 4_C study are not included in Table II but are plotted in Figures 2, 3 and 4. No control studies were done immediately before the oral administration of uric acid in the case of 4_C, but the results of the 4_A and 4_B control studies were very similar to one another and therefore have been used as a baseline. It can be seen that the changes after oral uric acid, although slight, were in the same direction as those induced by the other dietary supplements. Four Gm. of uric acid a day in other subjects induced similar changes in plasma and urine uric acid.

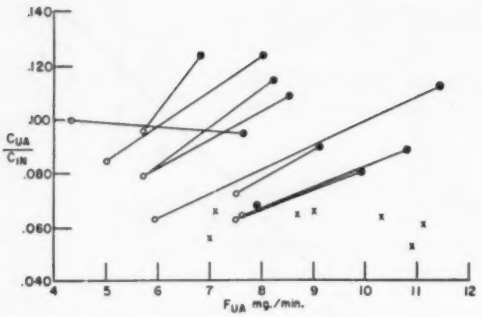

FIG. 3. THE RELATION OF THE PROPORTION OF FILTERED URIC ACID EXCRETED (C_{UA}/C_{IN}) TO THE FILTERED URIC ACID LOAD (F_{UA})

Symbol conventions are the same as in Figure 2. See text for discussion.

five days. No investigation was made of the role of variations in absorption or destruction in the gastrointestinal tract or variations in the degree of conversion to uric acid in determining the differences between the response to the various dietary regimens.

High purine intake in the nongouty subjects resulted in a rise of the mean plasma uric acid concentration to 7.9 mg. per 100 ml. Although, under these conditions, the mean filtered uric acid load was almost identical with the control value in the gouty patients, the nongouty subjects excreted uric acid at two times their control rate of excretion and their proportion of filtered uric acid excreted and their uric acid clearance far exceeded that of the gouty patients (Figure 1). The mean 24 hour urine uric acid excretion of the nongouty subjects rose well above their control level and exceeded the mean of the gouty patients.

Figure 2, in which renal tubular reabsorption of uric acid is plotted as a function of filtered uric acid load, reveals no gross difference between the gouty and the nongouty groups. The heavy diagonal line indicates total reabsorption of the filtered uric acid. The proportion of the filtered uric acid excreted is indicated by the vertical distance between a point and the heavy line compared to the vertical distance from the heavy line to the level of no reabsorption of uric acid. Some investigators have used similar data, plotted as in Figure 2, to support the notion that the percentage of the filtered load of urate reabsorbed in gout does not differ from that of normal subjects at equivalent filtered urate loads (1). Since only a very small proportion of the total filtered urate load is excreted either by gouty patients or by normal subjects, a plot of the data in this fashion tends to obscure differences in the proportion of filtered uric acid excreted. When the proportion of the filtered load of urate excreted (uric acid/inulin clearance ratio) is plotted as a function of filtered urate load (Figure 3), a difference is apparent between the nongouty subjects and the patients with gout. Under control conditions, the proportion of filtered uric acid excreted is slightly lower in the patients with gout than in the nongouty subjects. Oral administration of uric acid precursors to nongouty subjects resulted in the elevation of their filtered urate load to the range of those of the patients with gout. Under these circumstances the nongouty subjects excreted a higher proportion of their filtered urate load than did the patients with gout who had comparable filtered loads. All but one of the nongouty subjects excreted a higher proportion of their filtered load of uric acid during the experimental than during the control periods. The values for two gouty patients in Figure 3 lie in the area occupied by the nongouty subjects under control conditions. These two individuals, Patients 10 and 12, had the lowest plasma uric acid concentrations of the patients with gout. In addition, Patient 10 had the lowest inulin clearance among the patients with gout.

In Figure 4, the proportion of filtered uric acid excreted is plotted as a function of the plasma uric

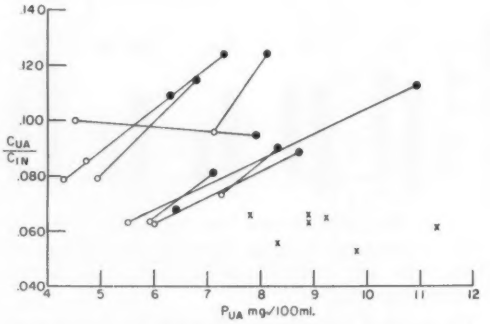

FIG. 4. THE RELATION OF THE PROPORTION OF FILTERED URIC ACID EXCRETED (C_{UA}/C_{IN}) TO THE PLASMA URIC ACID CONCENTRATION (P_{UA})

Symbol conventions are the same as in Figure 2.

acid concentration. The same general relations between the two groups shown in Figure 3 can be seen here. The control data of the nongouty subjects and gouty patients are better separated in Figure 4 than in Figure 3. Possibly the proportion of filtered urate excreted is a function of the plasma urate concentration rather than of the filtered load.

The results of the plasma uric acid determinations after protein precipitation differed from those reported in this paper only in that the plasma uric acid concentrations were 9 per cent lower, with a standard deviation of 4 per cent. This resulted in corresponding changes in the amounts of uric acid filtered and reabsorbed and in the uric acid clearance, but the relations between the various values and their appearance after plotting showed little difference from the results reported here.

DISCUSSION

It is usually stated that the plasma uric acid is completely filterable by the glomeruli, that a varying proportion of the filtered uric acid is reabsorbed by the renal tubules and that none is excreted by the tubules (16). There is considerable experimental evidence that plasma uric acid is completely filterable (17). As judged by the work of one group of investigators, who used paper electrophoresis with many different buffers and a wide variety of experimental conditions including variation in pH, ionic strength, temperature and potential gradients, no inhomogeneity of the plasma uric acid has been demonstrated (18). It is not so generally accepted that there is no renal tubular excretion of uric acid in humans. The anomalous decrease in the clearance of uric acid which uniformly occurs after the administration of small doses of a wide variety of agents which increase urate clearance at higher doses (1) is one of the facts which is difficult to explain if one accepts the view that uric acid is not excreted by the tubules. However, for convenience, and in the absence of conclusive evidence that the assumptions stated above are not correct, we will accept them for the purpose of this discussion.

Many investigations of renal function in gout have been carried out in the past. These have included study of the effect of feeding uric acid precursors (19). In these investigations, however, there has been great variation in the methods used for measurement of uric acid concentration and glomerular filtration rate. Because of their large number and the great differences in techniques employed, a discussion of all of the reports will not be presented here. The studies of Coombs and co-workers (5), of Talbott (6) and of Gutman and Yü (1) were carried out on large numbers of patients and inulin was used for the determination of glomerular filtration rates. In particular, the studies of Gutman and Yü (1) seem most suitable for comparison with our own. These investigators corrected for nonurate chromogen in urine by using uricase and demonstrated agreement of their colorimetric method with enzymatic spectrophotometric measurements (20). Some of the results of Gutman and Yü's studies are shown in Table III for comparison with the data of the present investigation.

The results of the renal function studies under control conditions reported here are similar to the findings of Coombs and associates (5), Talbott (6) and Gutman and Yü (1). The means of the uric acid clearance, proportion of filtered uric acid excreted and rate of uric acid excretion are only slightly different in the nongouty and gouty groups and there is some overlap of individual values. These findings have led many observers in the past to conclude that there is no abnormality in the renal excretion of uric acid by patients with gout (1, 5, 21). Oral purine loading of the nongouty subjects enabled us to study renal function in nongouty subjects and in gouty patients at comparable plasma uric acid concentrations. Under these conditions, the uric acid clearance, proportion of filtered uric acid excreted and rate of uric acid excretion by the nongouty subjects were well above the levels observed in the gouty patients. Since the filtered uric acid load of the nongouty subjects after purine loading was approximately the same as that of the gouty patients, the difference in renal excretion of uric acid between the two groups must be attributed to a lower tubular reabsorption of uric acid by the nongouty subjects.

The rise in 24 hour urine uric acid excretion in the nongouty subjects after high purine intake, although large, is not as great as one would expect from the doubling of the rate of uric acid excretion during the renal clearance studies. Several

TABLE III

*Renal function**

	Data of Gutman and Yü (1)		Present data		
			Nongouty males		
	Nongouty males	Gout	Control	Urate load	Gout
P_{UA} (*mg./100 ml.*)	5.6 ±1.3	9.0 ±1.4	5.6 ±1.1	7.9 ±1.3	9.2 ±1.1
C_{IN} (*ml./min.*)		98.5 ±17.7	110 ±17	114 ±18	100 ±11
C_{UA} (*ml./min.*)	8.7 ±2.5	7.5 ±2.4	8.5 ±1.2	11.7 ±1.9	6.2 ±0.8
F_{UA} (*mg./min.*)	6.3 ±1.4	10.1 ±2.8	6.1 ±1.2	8.9 ±1.5	9.2 ±1.7
T_{UA} (*mg./min.*)	5.8 ±1.4	9.5 ±2.6	5.6 ±1.1	8.0 ±1.4	8.6 ±1.6
C_{UA}/C_{IN}	0.076 ±0.024	0.068 ±0.021	0.078 ±0.014	0.104 ±0.016	0.062 ±0.005
$U_{UA}V$ (*mg./min.*)	0.493 ±0.159	0.663 ±0.235	0.46 ±0.06	0.92 ±0.17	0.57 ±0.10
24 Hr. Ur UA (*mg.*)	418 ±70	497 (range, 170 to 1,520)	527 ±91	926 ±260	647 ±227

* The results are expressed as the means and the standard deviations. The abbreviations are the same as those used in Table II.

explanations seem plausible. There are many extraneous factors which influence the excretion of uric acid. One would expect dietary changes (22, 23) and exercise (24, 25) to be more prominent variables during a 24 hour urine collection period than during a renal clearance study. One of these effects is strikingly illustrated in Subject 7. This subject hiked in mountainous country during the day of his urine collection period after purine loading. The prolonged and striking effect of exercise in decreasing the excretion of uric acid may well account for the relatively small increase in 24 hour urine uric acid excretion that was associated with a marked rise in plasma uric acid concentration and rate of uric acid excretion during the final renal function study in this subject.

The nongouty subjects were fed uric acid or its precursors for periods of only three days. It is conceivable that in time the normal subjects would have "adapted" to prolonged elevation of their plasma uric acid concentration and eventually would have excreted a smaller proportion of filtered uric acid. Repeated renal function studies were done on four nongouty subjects maintained for periods of one month on 4 to 8 Gm. of ribonucleic acid per day (26). There was no significant change in their renal excretion of uric acid associated with the passage of time.

In the past few years overproduction of uric acid has been regarded by many investigators as the basis for hyperuricemia in gout (1, 27, 28). The presence of tophaceous deposits around joints, uric acid calculi and large amounts of uric acid in the urine of some patients with gout led Garrod to conclude that "an undue formation of this substance would favor the occurrence of the disease" (29). However, there are many patients with gout in whom overproduction of uric acid is not readily apparent. The majority of gouty patients do not excrete abnormally large amounts of urate in their urine, many do not have tophi (1), and in some patients without tophi the miscible uric acid pool is only 3 to 5 Gm. (30, 31). The prolonged increase in urate excretion induced by administration of uricosuric agents to some patients with nontophaceous gout suggests that these patients have urate deposits that are not clinically obvious (32). The increased incorporation of

glycine into uric acid demonstrable in some patients with gout has been regarded as supporting the overproduction theory as the basis for hyperuricemia. In discussing overincorporation, Wyngaarden makes clear the distinction between early overincorporation of glycine into urinary uric acid, a defect actually demonstrated in some gouty patients, and overproduction of uric acid. Early overincorporation of glycine and significant overproduction of uric acid are not necessary concomitants (28).

Regardless of whether overproduction of uric acid occurs in all patients with gout, the data of the present investigation do not support the overproduction theory as the exclusive basis for hyperuricemia in gout. In the present study, the nongouty subjects required oral purines in amounts great enough to double their milligram per minute excretion rate of urates and far exceed the excretion rate of the gouty patients in order to produce hyperuricemia of similar magnitude to that observed in the gouty patients. Excluding individuals with renal disease, if the elevation of the serum uric acid in gout were simply due to overproduction of uric acid, then gouty patients should uniformly excrete larger than normal amounts of uric acid in their urine.

It seems very likely that gout is fundamentally the result of a metabolic abnormality. In view of the present facts, however, it does not seem likely that the hyperuricemia of most patients with gout is solely attributable to overproduction of uric acid. Abnormal renal excretion of uric acid is an important cause of hyperuricemia in some patients with gout. In addition to the overproduction of uric acid demonstrable in some patients, a metabolic defect in gout might affect the plasma urate concentration by: *a*) the production of a nonurate substance affecting renal excretion of uric acid and resulting in hyperuricemia; *b*) the production of uric acid that was bound to protein or differed from the remainder of the plasma uric acid in some other respect; or *c*) a metabolic defect might affect the kidneys in some more direct manner. We have no evidence to support any of these possibilities.

CONCLUSIONS

1. Renal function studies were performed on seven nongouty male subjects on 10 occasions and on six gouty male patients on seven occasions under control conditions. The inulin and uric acid clearances, proportion of filtered uric acid excreted and rate of uric acid excretion were similar in the two groups.

2. Uric acid precursors were administered orally to the nongouty subjects for three to five day periods in order to increase their plasma uric acid concentrations and filtered uric acid loads to the range found in the patients with gout. Under these conditions, the nongouty subjects reabsorbed a smaller proportion of their filtered uric acid and achieved a uric acid clearance and uric acid excretion rate well above the levels observed in the gouty patients.

3. It is concluded that abnormal renal excretion of uric acid is one important cause of hyperuricemia in some patients with gout.

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METABOLISM OF L-THYROXINE BY HUMAN TISSUE SLICES*

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(Submitted for publication May 11, 1959; accepted July 6, 1959)

The deiodination of L-thyroxine to triiodothyronine has been observed *in vitro* using surviving rat kidney slices (1, 2). Surveys of other rat tissues have not shown consistent deiodination of thyroxine, but occasional appearances of trace quantities of triiodothyronine in surviving heart slices and diaphragm (3) have led to the belief that other tissues in the rat convert thyroxine to triiodothyronine at a slower rate than kidney.

Relevance of these observations to human physiology might be questioned on the ground that conversion of L-thyroxine to triiodothyronine by extrathyroidal tissues is peculiar to the rat. In this connection, a previous report (4) that deiodination of L-thyroxine occurred in an athyreotic human has been retracted (5). It therefore seemed pertinent to examine the L-thyroxine deiodinating enzyme system of extrathyroidal tissues of the human. The purpose of this paper is to report such experiments employing tissue slices of human kidney, liver, skeletal muscle and heart.

METHODS

Materials and preparation. Samples of human kidney, liver, heart and skeletal muscle were obtained at operation. Adequate quantities of normal kidney tissue were available from nephrectomy in patients with renal carcinoma. Wedge biopsies of liver were carried out during laparotomy. Intercostal or rectus muscle samples were obtained during thoracotomy or laparotomy, and limited quantities of left auricular appendage were available during mitral commissurotomy.

The isolated tissue was immediately placed in chilled Krebs-Ringer phosphate solution. Slices (300 to 500 mg. wet weight) were prepared using a Stadie-Riggs apparatus.

Reaction procedure. The reaction mixture (final volume 3 ml.) consisted of Krebs-Ringer phosphate solution (pH 7.4) to which 0.01 μg. of I^{131}-labeled L-thyroxine[1] was added. Incubation was carried out at 37° C. for six hours. Tissue slices boiled for 10 minutes were used as a control.

Extraction and separation of products. At the end of the period of incubation the slices were removed, rinsed and homogenized in 2 ml. of water. The homogenates were extracted with 10 volumes of 3 per cent concentrated ammonium hydroxide in butanol. The extracts were concentrated to a small volume, marker thyronine compounds were added and the sample dried on Whatman No. 3 mm. paper for chromatography. Chromatograms were run in descent for 24 hours using a tertiary amyl alcohol—2 N ammonium hydroxide solvent system. The radioactive compounds were located on the strips with a continuously recording automatic scanner. The position of the marker compounds was determined by color development with 4-amino-antipyrine reagent.[2] With the solvent system used, the R_f values of the thyronine compounds in simple solution are: thyroxine, 0.20; tetraiodothyroacetic acid, 0.30; triiodothyronine, 0.40 and triiodothyroacetic acid, 0.56. The presence of extraneous material derived from butanol extraction of the tissues has a tendency to proportionally lower the R_f values, as seen in Figure 1. For this reason, marker compounds are always added.

A portion of the thyronine compounds is lost during the extraction procedure. Approximately 60 per cent of the radioactivity added as thyroxine appears on the chromatogram. For this reason, the quantity of triiodothyronine derived from thyroxine can only be estimated by comparing the relative quantities of thyroxine and triiodothyronine on the chromatogram. This is done by measuring the area under the radioactivity curves. Recognizing that thyroxine is randomly labeled in the 3′ and 5′ positions and that the incidence of double labeling is negligible, it follows that the labeled triiodothyronine represents only one-half of the labeled thyroxine present on the chromatogram. This may be expressed as a per

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[1] Obtained from Abbott Laboratories.

[2] The application of the 4-amino-antipyrine reagent (6) to the detection of thyronine compounds and their acetic acid analogues was suggested by Dr. Leon Saunders, Smith, Kline and French Laboratories. The chromatogram is first sprayed with a 2 per cent solution of 4-amino-antipyrine in 2 per cent sodium carbonate and then with a 2 per cent solution of potassium ferricyanide. Tetraiodinated compounds give a lavender, triiodinated a red and diiodinated an orange color. The method is somewhat more sensitive than the diazo method previously used in this laboratory.

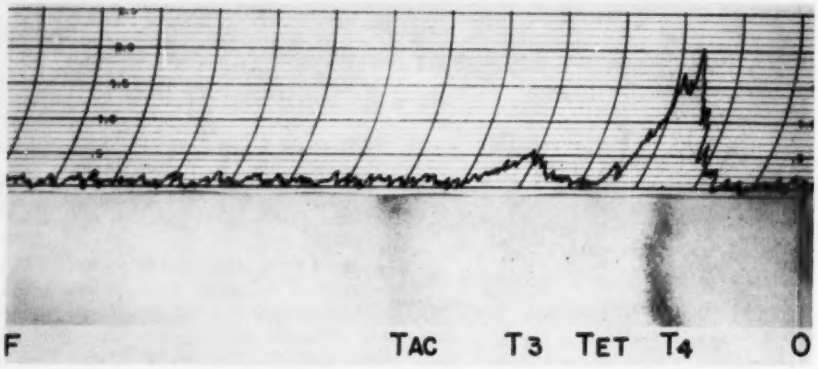

FIG. 1. RADIOCHROMATOGRAM FROM HUMAN KIDNEY SLICE EXPERIMENT SHOWING DEIODINATION OF I^{131}-LABELED L-THYROXINE TO TRIIODOTHYRONINE

Note separation of triiodothyronine from tetrac. O = origin of chromatogram, F = solvent front. Position of marker compounds: T_4, thyroxine; TET, tetraiodothyroacetic acid; T_3, triiodothyronine; TAC, triiodothyroacetic acid.

cent as follows:

$$\text{per cent triiodothyronine derived from thyroxine} = \frac{2 \times \text{triiodothyronine radioactivity}}{2 \times \text{triiodothyronine radioactivity} + \text{thyroxine radioactivity}} \times 100.$$

RESULTS

With kidney slices radioactivity regularly appeared at the position of triiodothyronine. The extent of conversion of thyroxine to triiodothyronine ranged from 11 to 38 per cent (mean 22 per cent) (Table I) in 10 consecutive experiments. Iodide liberated by the deiodinating system does not appear on the chromatogram due to its partial loss into the reaction mixture during incubation and to the unfavorable partition ratio (10:1, water to butanol) during ammoniacal butanol extraction of the residue. A representative experiment is shown in Figure 2, demonstrating the appearance of triiodothyronine in the viable tissue and its absence in the boiled control. A separate experiment is shown in Figure 1 in which, in addition to triiodothyronine, tetraiodothyroacetic acid and triiodothyroacetic acid were added as marker compounds. Separation of tetraiodothyroacetic acid from triiodothyronine is demonstrated. The only labeled reaction product observed is triiodothyronine.[3]

Tissues other than kidney showed little or no deiodination as illustrated in Figure 3. In an occasional experiment with heart slices, a small peak appeared at the triiodothyronine position but this finding was sufficient neither in quantity nor consistency to establish the reaction in this tissue.

TABLE I

The extent of conversion of I^{131}-labeled L-thyroxine to triiodothyronine by human kidney slices

Experiment No.	Per cent
1	16
2	11
3	29
4	15
5	18
6	16
7	38
8	34
9	26
10	20

DISCUSSION

Two physiological origins of triiodothyronine have been proposed: *1*) the coupling of diiodotyrosine and monoiodotyrosine in the thyroid and

[3] The labeled reaction product from thyroxine substrate is considered not to be tetraiodothyroacetic acid for several reasons: the separation of triiodothyronine and tetraiodothyroacetic acid with the solvent system used is very satisfactory (Figure 1), the color of the compounds with 4-amino-antipyrine is distinguishable (Footnote 2), and with slice preparations tetraiodothyroacetic acid has not been encountered, whereas it has been observed using mitochondrial enzyme systems.

its secretion as such from the gland, and *2*) the monodeiodination of thyroxine in extrathyroidal tissues.

There is general agreement that triiodothyronine is formed in the thyroid, and there is evidence that circulating triiodothyronine is derived from this source rather than from deiodination of thyroxine in peripheral tissues. The thyroidal vein of animals was found to contain more triiodothyronine than is present in the general body circulation (*7*), and patients with no functioning thyroid tissue did not have significant plasma levels of labeled triiodothyronine following the administration of labeled thyroxine (5). Since cells have a greater affinity for triiodothyronine than for thyroxine, it would be surprising if triiodothyronine formed intracellularly were released into the circulation. The low concentration of triiodothyronine in plasma relative to thyroxine does not necessarily reflect the quantitative output from the gland. Labeled triiodothyronine injected intravenously rapidly leaves this compartment and appears in tissues while thyroxine, preferentially protein-bound, is maintained at higher levels in the intravascular compartment.

Production of triiodothyronine by the gland does not exclude its alternative origin from thyroxine in target tissues. It is quite possible that both mechanisms are operative. The proposal of Gross and Pitt-Rivers (8) that the conversion of thyroxine to triiodothyronine is an essential preliminary step in the "activation" of thyroid hormone is supported by several observations: *1*) the relatively longer latent period between administration of thyroxine and appearance of maximal metabolic effects than is observed with triiodothyronine; *2*) recovery of a labeled compound subsequently identified as triiodothyronine from plasma and tissues following injection of labeled thyroxine in thyroidectomized mice (9) or propylthiouracil-treated rats (10); *3*) demonstration of deiodination of thyroxine to triiodothyronine *in vitro* by surviving rat kidney slices (1); *4*) the direct relationship between the rate of the deiodination re-

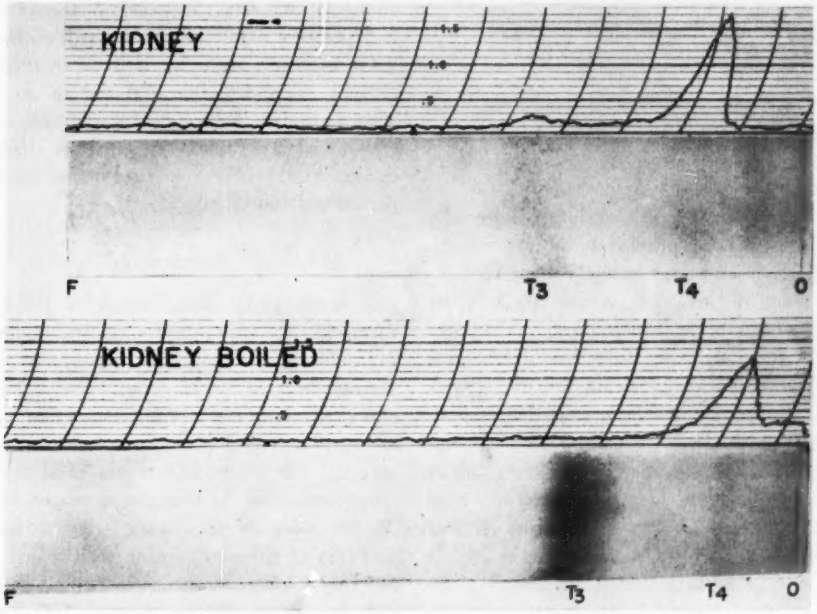

FIG. 2. RADIOCHROMATOGRAM FROM HUMAN KIDNEY SLICE EXPERIMENT SHOWING DEIODINATION OF I^{131}-LABELED L-THYROXINE TO TRIIODOTHYRONINE WITH SURVIVING TISSUE AND THE ABSENCE OF LABELED TRIIODOTHYRONINE WITH TISSUE INACTIVATED BY BOILING

O = origin of chromatogram, F = solvent front. Position of marker compounds: T_4, thyroxine; T_3, triiodothyronine.

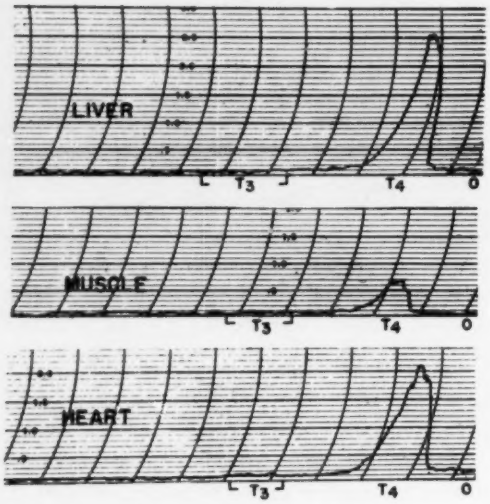

FIG. 3. RADIOACTIVITY RECORDS FROM CHROMATOGRAMS OF HUMAN LIVER, SKELETAL MUSCLE AND HEART EXPERIMENTS WITH I^{131}-LABELED L-THYROXINE AS SUBSTRATE

Note the absence of triiodothyronine peak with liver and muscle. O = origin of chromatogram. Solvent front not shown. Position of marker compounds: T_4, thyroxine; T_3, triiodothyronine.

action and the level of thyroid activity (11); and 5) the failure of D-thyroxine, which has little or no metabolic activity, to undergo deiodination in these *in vitro* systems (12).

The results obtained in this study are essentially the same as observed previously in rat tissues. Kidney regularly and most actively deiodinates L-thyroxine to form triiodothyronine while other tissues show little or no deiodination. Occasional appearance of trace quantities of triiodothyronine has been observed with rat heart and diaphragm, and a similar finding has been noted in experiments with human heart (Figure 3). This suggests the possibility that tissues other than kidney may convert thyroxine to triiodothyronine at a much slower rate. The reason for the greater activity of this enzyme system in the kidney is unknown. It is unlikely that it represents a degradative reaction related to the excretory function of this organ since triiodothyronine is more active than thyroxine. More plausible is the possibility that it is related to the high metabolic rate of this tissue. The Qo_2 of kidney is reported to be three to four times as great as liver, five to six times as great as skeletal muscle and seven to eight times as great as heart (13). *In situ* activation of thyroxine by conversion to triiodothyronine at different rates would permit extrathyroidal tissues to control the level of hormone stimulation locally.

Technical considerations offer difficulty in demonstrating triiodothyronine formation by tissues having a less active deiodinating enzyme system than the kidney. Based on the daily consumption of 200 μg. of thyroxine by a 70 Kg. man, and assuming that all of it is converted to triiodothyronine by extrathyroidal tissues and that all tissues carry out the transformation at the same rate, approximately 0.001 μg. of thyroxine would be deiodinated to triiodothyronine per G. of tissue per six hours by any tissue. In the present experiments the weight of tissue used was in the range of 300 to 500 mg. We would expect, therefore, that with the above assumptions only 0.0003 to 0.0005 μg. of thyroxine would be converted to triiodothyronine. Since 0.01 μg. of thyroxine was added to each flask, the average yield would be 3 to 5 per cent of the substrate used, a quantity difficult to detect even by isotopic methods. It is evident, therefore, that only in tissue in which deiodination of thyroxine to triiodothyronine occurs at a rate considerably higher than average can triiodothyronine be observed under these experimental conditions.

SUMMARY

The enzymatic deiodination of I^{131}-labeled L-thyroxine to triiodothyronine by human extrathyroidal tissues has been examined using surviving slices prepared from kidney, heart, liver and skeletal muscle. With kidney, the appearance of labeled triiodothyronine was regularly observed. The extent of conversion of thyroxine to triiodothyronine by this tissue ranged from 11 to 38 per cent in six hours. With heart, trace amounts of triiodothyronine were noted occasionally, but in quantity insufficient to definitely establish the reaction in this tissue. Triiodothyronine formation was not observed in liver and muscle. It is suggested that the greater activity of the thyroxine-deiodinating enzyme in kidney is related to the high metabolic rate of this tissue.

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THE PLASMA VITAMIN B_{12} BINDING SUBSTANCE: I. ITS DETECTION IN THE SEROMUCOID FRACTION OF PLASMA FROM NORMAL SUBJECTS AND PATIENTS WITH CHRONIC MYELOCYTIC LEUKEMIA*

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It is well known that the striking increases in serum vitamin B_{12} concentrations seen in patients with chronic myelocytic leukemia (CML) are associated with an increased plasma binding capacity for B_{12}. The latter has been demonstrated *in vitro* (1–4) using microbiologic assay or dialysis. *In vivo* studies in patients with CML have demonstrated a delay in the plasma disappearance of either an intravenously (5, 6) or orally (7) administered dose of $Co^{58}B_{12}$ and would appear to confirm the *in vitro* findings.

The association of a high serum B_{12} with a high plasma binding capacity has stimulated investigation into the nature of the plasma binding substance. Previous attempts to characterize the B_{12} binding substance have been primarily concerned with defining its electrophoretic mobility, and thus far actual isolation and chemical characterization have not been achieved (1, 8–10). The fact that B_{12} apparently exists in a macromolecular complex at several stages of its metabolism, including gastrointestinal absorption (11), plasma transport (1) and liver storage (12), led us to consider whether or not a similar type of substance binds B_{12} in each case. Since intrinsic factor, which plays a key role in the gastrointestinal absorption of B_{12}, is thought to be a glycoprotein (13), we undertook the present studies to determine whether or not the B_{12} binding substance of plasma is also a glycoprotein.

Co^{58} vitamin B_{12} was used to label the serum B_{12} binding protein, either *in vitro* or *in vivo*. Glycoproteins were isolated according to modifications of the methods of Winzler, Weimer, and co-workers (14, 15). The results obtained with plasma from patients with CML were compared to those obtained with plasmas from subjects having normal serum B_{12} concentrations. The latter were either normal bank blood donors or patients with malignant disease and normal serum B_{12} concentrations, and in the text are referred to as "control" subjects.

METHODS

Microbiologic assay. A modification described in detail elsewhere (16) of the U. S. P. method using *Lactobacillus leichmannii*, American Type Culture Collection No. 7830, was utilized in measuring the B_{12} concentrations of sera and serum fractions. The mean and standard error of B_{12} levels determined by this method in 31 normal subjects was 0.533 ± 0.030 mμg. per ml.[1]

Radioactivity assay. The $Co^{58}B_{12}$ had an original specific activity of 2.38 μc. per μg.[2] One mμg. of this material gave approximately 1,900 cpm above a background of 180 cpm. All counting was done in a well-type scintillation counter. Samples were counted for a period of time sufficient to give a counting error of less than 3 per cent.

Biologic activity of the labeled vitamin was confirmed by *L. leichmannii* assay and found to be 93 per cent of that given by the manufacturer. Suitable dilutions were made with the buffer described below.

Perchloric-phosphotungstic acid precipitation. The *in vitro* studies were done as follows. To 3 ml. aliquots of plasmas in dialysis bags was added 1 ml. of solutions containing sufficient $Co^{58}B_{12}$ to provide concentrations ranging from 0.1 to 100 mμg. of $Co^{58}B_{12}$ per ml. of plasma. The bags were tied, and the contents mixed by inversion and incubated at room temperature for one hour. Each bag was placed in a 125 ml. Erlenmeyer flask and 100 ml. of buffer was added.[3] Dialysis was performed for a total

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[1] The abbreviation mμg. as used in this paper means 10^{-9} gram.

[2] Purchased from Merck and Co., Inc., Rahway, N. J.

[3] The buffer used was 0.15 M sodium phosphate adjusted to pH 7.3 and diluted with nine parts of 0.85 per cent sodium chloride.

of 72 hours at 4° C. with replacements of the buffer at 24 and 48 hours. The contents of each dialysis bag was transferred to a 4 ml. counting vial and counted against a standard. Each vial was then emptied into a 50 ml. beaker to which was added 18 ml. of 0.85 per cent saline. The saline facilitated rinsing of the vial and served to dilute out the glycoproteins, thus minimizing loss by coprecipitation with the perchloric acid precipitable proteins. Ten ml. of 1.8 M perchloric acid was slowly added to each beaker with mixing and five minutes later the contents filtered through Whatman No. 12 folded filter paper. The dried filter paper with its precipitate was folded and transferred to a 4 ml. counting vial. The clear perchloric acid filtrate was collected in a 50 ml. centrifuge tube, 5.6 ml. of 5 per cent phosphotungstic acid in 2 N HCl was added and the contents were mixed by inversion. Five minutes later this mixture was centrifuged at 3,000 rpm for 30 minutes. The clear supernatant was decanted and a 4 ml. aliquot assayed for radioactivity. The phosphotungstic acid precipitate was redissolved in 4 ml. of 1 N NaOH and also assayed for radioactivity.

Calculations were made as follows:

A. per cent bound to whole plasma =

$$\frac{\text{cpm whole plasma after dialysis}}{\text{cpm added}} \times 100;$$

B. per cent bound to a protein fraction =

$$\frac{\text{cpm of that fraction}}{\text{cpm whole plasma after dialysis}} \times 100;$$

C. mμg. bound to a protein fraction =

$$\frac{(\text{m}\mu\text{g. } Co^{58}B_{12} \text{ originally added})\ (A)}{10{,}000}\ (B);$$

D. per cent recovery =

$$\frac{\text{sum of cpm from each protein fraction}}{\text{cpm whole plasma after dialysis}} \times 100.$$

"MP-1" isolation. Plasma was fractionated according to a modification of the method of Weimer, Mehl and Winzler (15). In the *in vitro* studies, 1 ml. of a $Co^{58}B_{12}$ solution containing 20 mμg. per ml. was added to 20 ml. of plasma (1 mμg. $Co^{58}B_{12}$ per ml. plasma) and the mixture incubated at 4° C. for one hour. In the *in vivo* studies, endogenous labeling of plasma with radioactive $Co^{58}B_{12}$ was carried out as described in the section on *in vivo labeling.*

To one volume of plasma, previously labeled with $Co^{58}B_{12}$ in either of the above manners, 1.2 volumes of saturated $(NH_4)_2SO_4$ were slowly added with mixing. After this mixture had stood at 4° C. for 16 hours, a precipitate ("Ppt. A") was separated by centrifugation for 30 minutes at 2,000 rpm. The supernatant was decanted, reduced to pH 4.75 with 1 N HCl and allowed to stand for 16 hours at 4° C. A precipitate ("Ppt. B") was separated by centrifugation as before and the supernatant was reduced to pH 3.7 with 1 N HCl. After this supernatant had stood for another 16 hours at 4° C., a precipitate ("Ppt. C") was again separated by centrifugation. The supernatant was saturated with $(NH_4)_2SO_4$ and the "MP-1" fraction thereby precipitated. This precipitation was facilitated by transferring the pH 3.7 supernatant to a dialysis sac which was then suspended in a beaker containing saturated $(NH_4)_2SO_4$ with an excess of the solid salt. After the sac had remained suspended in solution for 72 hours at 4° C., a precipitate ("MP-1") formed in the sac and subsequently was separated by centrifugation. Precipitates A and B, which contained the bulk of the added radioactivity, were redissolved in water and treated with perchloric and phosphotungstic acid in a manner similar to that described above. The resulting precipitates were assayed for radioactivity. Calculations were performed as described above.

In vivo labeling. After overnight fasts, patients were given 0.57 μg. of $Co^{58}B_{12}$ by mouth and fed one hour later. Blood samples were withdrawn into heparinized syringes at three hour intervals up to 12 hours, then at 24 and 36 hours. After assaying the radioactivity in 4 ml. of whole plasma from each sample, 10 to 20 ml. of plasma from the sample having the highest cpm was fractionated according to the previously described methods. The cpm in each fraction were expressed as the per cent of cpm present in whole plasma.

RESULTS

A. Perchloric-phosphotungstic acid method

1. In vitro addition of $Co^{58}B_{12}$. The results obtained by perchloric-phosphotungstic acid precipitation of plasmas (to which 0.120 mμg. $Co^{58}B_{12}$ per ml. had been added) obtained from five subjects with normal serum B_{12} concentrations and five patients with CML and increased B_{12} concentrations are listed in Table I. The phosphotungstic acid precipitates contained a mean of 78 per cent of added $Co^{58}B_{12}$ for the control group and a mean of 77 per cent for the CML group. Perchloric acid precipitates contained a mean of 17 per cent and 11 per cent for the two groups, respectively. Total recovery ranged from 80 to 113 per cent of the added material. The dialysates contained no radioactivity indicating practically complete binding at this concentration.

2. Binding of $Co^{58}Cl_2$. In order to exclude the possibility that radioactivity present in each fraction represented free cobalt, the binding characteristics of $Co^{58}Cl_2$ were studied.[4]

When $Co^{58}Cl_2$ equivalent to the amount of Co^{58} present in 1 mμg. of $Co^{58}B_{12}$ was added to plasma, and a procedure otherwise identical to that used

[4] $Co^{58}Cl_2$ obtained from Oak Ridge National Laboratory was carrier-free and had an original concentration of 1.26 mc. per ml.

TABLE I

In vitro addition of 0.600 mμg. $Co^{58}B_{12}$ per 5 ml. of plasma (0.120 mμg. per ml.) followed by perchloric-phosphotungstic acid precipitation

Subject	Diagnosis	Serum B_{12}	Per cent of added $Co^{58}B_{12}$		
			In perchloric acid Ppt.	In phosphotungstic acid Ppt.	Recovery
		mμg./ml.			
I. Normal B_{12}					
A	Normal	0.461	16	79	95
B	Normal	0.265	13	71	84
J. Q.	Partial resection of ileum	0.299	18	95	113
O. C.	Prostatic carcinoma	0.353	17	80	97
D. S.	Embryonal rhabdomyosarcoma	0.497	20	66	86
Mean			17	78	95
II. High B_{12}					
L. R.	Chronic myelocytic leukemia	1.200	6	83	89
M. B.	Chronic myelocytic leukemia	2.681	7	78	85
O. F.	Chronic myelocytic leukemia	7.825	8	72	80
O. F.	Chronic myelocytic leukemia	7.983	13	73	86
J. S.	Chronic myelocytic leukemia	13.475	23	81	104
Mean			11	77	89

for $Co^{58}B_{12}$ was followed, the recoveries of added material were as follows:

	Per cent of cpm added
Whole plasma (after dialysis)	22.2
Phosphotungstic acid precipitate	1.89
Perchloric acid precipitate	8.84

thus demonstrating that with equal amounts of Co^{58}, at the concentrations indicated, that which is added as $Co^{58}Cl_2$ is not as effectively bound to whole plasma as is B_{12} (22 vs. 95 per cent), and that in contrast to B_{12}, most of the Co^{58} is found in the perchloric acid precipitate. The fact that the sum of the material recovered in the two precipitates does not equal that which was present in the dialyzed plasma may indicate that perchloric acid had freed some of the bound Co^{58} in the case of $Co^{58}Cl_2$.

3. Precipitation in the presence of an excess of nonradioactive B_{12}. Because of the possibility that the appearance of B_{12} in the phosphotungstic acid precipitate of a perchloric acid filtrate represented an artifact introduced by the action of perchloric acid on the original binding protein, by causing release of the bound vitamin, radioactive B_{12} was allowed to bind with whole plasma, and the subsequent perchloric and phosphotungstic acid precipitations were carried out in the presence of an excess of nonradioactive B_{12}.

Two 3 ml. aliquots of plasma were combined with 0.1 mμg. of $Co^{58}B_{12}$ per ml. plasma. Both were processed in the same manner (see Methods), but for the fact that following dialysis and prior to the addition of perchloric acid, 100 mμg. of nonradioactive B_{12} per ml. of plasma was added to Sample "A." Recovery of radioactivity in the phosphotungstic acid precipitate was not influenced by the presence of an excess of nonradioactive B_{12}.

	$Co^{58}B_{12}$ mμg./ml. plasma	Nonradioactive B_{12} mμg./ml. plasma	Recovery of $Co^{58}B_{12}$: Perchloric acid Ppt. %	Recovery of $Co^{58}B_{12}$: Phosphotungstic acid Ppt. %
A	0.1	100	16.7	74.4
B	0.1	0	12.0	71.4

4. In vitro addition of increasing amounts of $Co^{58}B_{12}$ to normal and CML plasma. The preceding studies suggest that most of the $Co^{58}B_{12}$ added to plasma at a concentration of 0.120 mμg. per ml. can be recovered in the phosphotungstic acid precipitate. These experiments compare the results obtained with the addition of increasing amounts of B_{12} to plasmas of five normal bank blood donors to that obtained on the plasma from eight patients with CML (Table II and Figures 1 and 2).

a. Bound to whole plasma (Table II). For all subjects studied, virtually all of the added $Co^{58}B_{12}$

TABLE II

In vitro addition of $Co^{58}B_{12}$; per cent bound to whole plasma and absolute amount in perchloric and phosphotungstic acid precipitates **

Subject	Serum B_{12}	WBC	Per cent bound to whole plasma: $Co^{58}B_{12}$ added (*mμg./ml.* plasma) 0.1	1.0	10.0	100.0	mμg. bound to protein fractions: $Co^{58}B_{12}$ added (*mμg./ml.* plasma) 0.1 Perch.	0.1 Phosph.	1.0 Perch.	1.0 Phosph.	10.0 Perch.	10.0 Phosph.	100.0 Perch.	100.0 Phosph.
	mμg./ml.													
Normals														
A	0.461		95	93	18	5	0.012	0.079	0.110	0.745	0.370	1.26	3.18	2.54
B	0.510		92	99	18	4	0.019	0.073	0.186	0.724	0.448	1.29	1.81	1.83
C	0.320			97	16	4			0.157	0.725	0.368	1.16	1.51	2.02
D	0.400			93		5			0.123	0.766			1.82	3.30
E	0.380			95	12				0.183	0.699	0.299	0.83		
Mean				95	16	5			0.110	0.732	0.288	1.135	2.08	2.42
CML														
D. L.	9.725	13,600		100	89	24			0.070	0.840	0.690	6.18	2.96	18.60
J. L.	8.700	5,300		92	50	8			0.097	0.764	0.640	4.06	1.91	5.03
O. F.	7.937	100,000		97	98	15			0.074	0.877	1.086	8.56	2.64	11.62
L. E.	7.800	20,000			86	12					0.907	7.33	2.09	9.66
J. S.	7.300	14,600			44	7					0.565	3.56	1.55	4.95
R. F.	2.033	52,000		95	22	4			0.090	0.770	0.420	1.67	1.37	2.32
M. B.	2.000	81,600			18	4					0.111	0.69	1.05	2.37
P. P.	1.875	7,200		88	10	3			0.160	0.720	0.240	0.73	1.01	1.42

* Per cent bound = cpm after dialysis/cpm added; perch. = perchloric acid precipitate; phosph. = phosphotungstic acid precipitate (seromucoid).

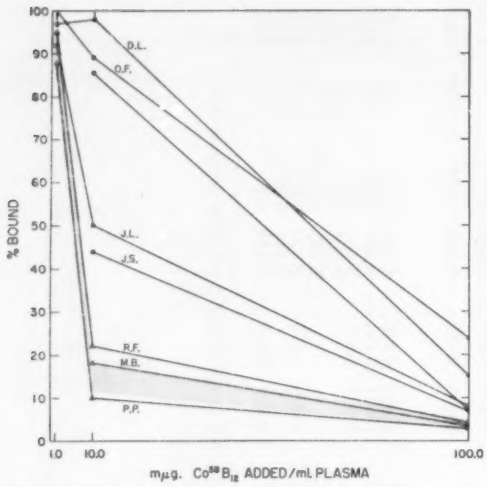

FIG. 1. PER CENT OF $Co^{58}B_{12}$ BOUND TO WHOLE PLASMA FOLLOWING *IN VITRO* ADDITION OF 1.0, 10.0 AND 100 MμG. PER ML. OF PLASMA AND DIALYSIS

The shaded area indicates the range in subjects with a normal serum B_{12}. Closed circles represent CML subjects with a serum B_{12} greater than 2.1 mμg. per ml.; closed triangles represent CML subjects with a serum B_{12} less than 2.1 mμg. per ml.

was nondialyzable (83 to 100 per cent) when added at a concentration of 0.1 or 1 mμg. per ml. plasma; with the addition of larger amounts, the per cent bound fell off sharply in the normal B_{12} concentration group, but remained high in those CML patients with a high serum B_{12}. At a concentration of 10 mμg. of added $Co^{58}B_{12}$ per ml. plasma, five normal subjects bound a mean of 16 per cent (range = 12 to 18) of the added material, whereas the eight patients with CML and a high B_{12} bound a mean of 47 per cent (range = 10 to 89). When 100 mμg. of $Co^{58}B_{12}$ was added per ml. of plasma, normals bound a mean of 4.5 per cent (range = 4 to 5) and CML patients bound 9.3 per cent (range = 3 to 24). Within the CML group those subjects with a higher B_{12} concentration bound a greater per cent of the added material than did those with a less striking elevation. Three subjects, R. F., M. B. and P. P., had a pattern similar to that of the normals. They had serum B_{12} concentrations in the 2.0 mμg. per ml. range. The other five CML subjects with increased binding had serum B_{12} concentrations in the 7.0 mμg. per ml. range.

b. Bound to phosphotungstic acid precipitate (*Table II*). The increased binding capacity for added $Co^{58}B_{12}$ by plasma from patients with CML was found to be associated with an increased recovery of radioactive material in the phosphotungstic acid precipitate. In the five subjects with CML whose plasma bound a greater than normal amount of $Co^{58}B_{12}$, the absolute amount of $Co^{58}B_{12}$ found in the phosphotungstic acid precipitate when 10 mμg. of $Co^{58}B_{12}$ per ml. of plasma was added, had a mean of 5.94 mμg. per ml. plasma, whereas normals at the same concentration of added $Co^{58}B_{12}$ had a mean of 1.14 mμg. per ml. in their phosphotungstic acid precipitate. At a concentration of 100 mμg. of $Co^{58}B_{12}$ per ml. of plasma, the five CML subjects had a mean of 10.01 mμg. per ml. in their phosphotungstic acid precipitate, whereas the normals had a mean of only 2.42 mμg. per ml. The three previously mentioned CML subjects in partial remission had a normal amount of $Co^{58}B_{12}$ bound in their phosphotungstic acid precipitate.

Inspection of Figure 2 demonstrates that in

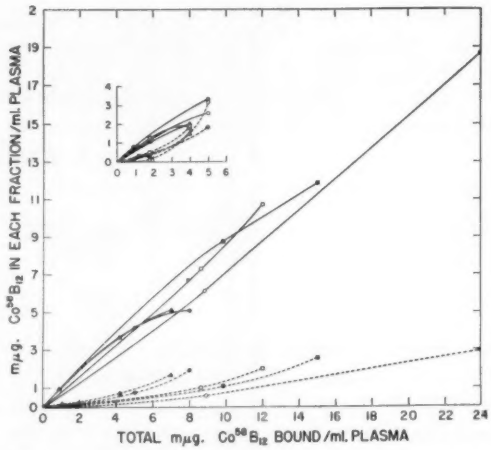

FIG. 2. THE ABSOLUTE AMOUNT OF $Co^{58}B_{12}$ BOUND TO PHOSPHOTUNGSTIC ACID PRECIPITATE, I.E., SEROMUCOID (SOLID LINE CURVES) AND PERCHLORIC ACID PRECIPITATE (INTERRUPTED LINE CURVES)

The main graph is on sera from five subjects with CML. The insert (same scale) is on five sera with a normal B_{12} concentration. "Total mμg. $Co^{58}B_{12}$ bound" is that present following dialysis. The three points on each subject's curve correspond to concentrations of 1, 10 and 100 mμg. per ml. plasma of added $Co^{58}B_{12}$.

TABLE III

*The per cent of $Co^{58}B_{12}$ recovered in various protein fractions, using the procedure for isolation of "MP–1"**

		MP–1 fractionation					Distribution of $Co^{58}B_{12}$ in subfractions of A and B					
		Distribution of $Co^{58}B_{12}$ in $(NH_4SO_4)_2$ fractions					Ppt. A			Ppt. B		
	Serum B_{12}	Ppt. A	Ppt. B	Ppt. C	MP–1	Total recovery	Perch.	Phosph.	Total recovery	Perch.	Phosph.	Total recovery
	mμg./ml.	%	%	%	%	%	%	%	%	%	%	%
Normals												
A	0.710	70.70	3.88	0.36	5.72	80.66	13.66	89.20	102.90	5.99	87.47	93.46
B	0.580	71.88	4.15	0.34	0.36	76.73	10.96	100.81	111.77	10.90	86.10	97.00
C	0.603	74.89	5.34	1.07	8.18	89.48	9.83	87.67	97.50	5.36	80.96	86.32
D	0.440	64.11	5.18	0.25	8.39	77.93	9.61	94.72	104.33	2.72	55.16	57.88
E	0.640	65.03	4.59	4.96	4.84	79.42	23.11	60.29	83.40	4.20	89.44	93.64
F	0.950	56.01	4.30	1.51	4.62	66.44	31.14	87.55	118.69	8.00	83.00	91.00
G	0.328	69.84	5.91	1.82	10.61	88.18	29.55	68.91	98.46	4.52	62.73	67.25
H	0.425	75.48	3.37	1.52	6.72	87.09	23.19	52.96	76.15	6.37	92.58	98.95
Mean		68.49	4.59	1.48	6.18	80.74	18.88	80.26	99.15	6.01	79.68	85.68
CML												
D. L.	17.150	26.28	23.55	2.21	18.90	70.94	8.04	83.15	91.19	4.58	76.96	81.54
J. L.	8.700	30.04	19.16	17.58	5.01	71.79	6.47	66.37	72.84	6.14	83.95	90.09
J. S.	7.300	51.03	15.72	1.52	9.28	77.65	8.21	78.46	86.67	6.97	73.58	80.55
R. F.	1.975	56.85	17.70	1.18	11.22	85.77	9.62	70.02	79.64	5.66	83.43	89.09
M. B.	1.550	65.89	9.24	0.57	9.56	85.26	15.29	75.07	90.36	5.17	63.22	68.39
P. P.	1.187	67.86	9.80	0.78	7.89	86.33	14.63	83.56	98.19	9.57	73.17	82.74
Mean		49.65	15.86	3.97	10.31	79.64	10.38	76.11	86.49	6.35	75.72	82.09

* $Co^{58}B_{12}$ added *in vitro* (1 mμg. per ml.); ppts. A, B, C and MP–1, see methods; perch. = perchloric acid precipitate; phosph. = phosphotungstic acid precipitate.

plasma from subjects with a normal serum B_{12} concentration, when more than 2 mμg. of $Co^{58}B_{12}$ is bound per ml. of whole plasma, the per cent of total bound vitamin recovered in the phosphotungstic acid precipitate falls off, whereas that in the perchloric acid precipitate rises. In contrast, in none of the eight subjects with CML did the per cent of bound B_{12} present in the phosphotungstic acid precipitate decrease until bound B_{12} exceeded 4 mμg. per ml. of plasma, and in two, the per cent did not fall off until bound B_{12} exceeded 8 mμg. per ml. of plasma.

5. In vivo labeling. Plasma from two subjects, previously labeled *in vivo,* was fractionated according to the perchloric-phosphotungstic acid method. The results were as follows:

D. S. (embryonal rhabdomyosarcoma)

	Serum B_{12} = 0.478 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (10 ml.)	100	
Perchloric acid Ppt.	0	0
Phosphotungstic acid Ppt.	73	73

J. L. (CML)

	Serum B_{12} = 8.225 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (20 ml.)	520	
Perchloric acid Ppt.	0	0
Phosphotungstic acid Ppt.	303	58.3

B. MP-1 fractionation

The fact that most of the radioactive B_{12} bound to plasma either *in vitro* or *in vivo* could be recovered in the phosphotungstic acid precipitate of a perchloric acid filtrate in both normals and CML's indicated that the major plasma B_{12} binding substance was present in the "seromucoid fraction" of plasma. It was, therefore, of interest to determine whether or not the substance was identical with the "MP-1" fraction of seromucoid obtained by Weimer, Mehl and Winzler (15).

Plasma from eight normal bank blood donors and plasma from six patients with CML were labeled *in vitro* with $Co^{58}B_{12}$ (1 mμg. per ml. plasma). The plasmas were fractionated according to a modification of the MP-1 isolation procedure of Winzler and the per cent recovery of added $Co^{58}B_{12}$ in each fraction was determined. The results are listed in Table III and are summarized as follows:

	No. of subjects	Ppt. A	Ppt. B	Ppt. C	MP-1	Total recovery
Normals	8	68.49	4.59	1.48	6.18	80.74
CML's	6	49.65	15.86	3.97	10.31	79.64

They indicate that MP-1 is not the major B_{12} binding substance in either normals or CML's. Most of the radioactivity was found in Ppts. A and B. Subsequent treatment of each of these with perchloric acid and phosphotungstic acid resulted in good recovery of radioactivity (82 to 99 per cent) in the phosphotungstic acid precipitate thus further demonstrating the "seromucoid" nature of the binding substance (Table III).

Using the same techniques, fractionation of plasma labeled *in vivo* was carried out in two subjects:

D. S. (embryonal rhabdomyosarcoma)

	Serum B_{12} = 0.478 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (20 ml.)	200	
Ppt. A	120	60
Ppt. B	0	0
Ppt. C	0	0
MP-1	6	3
	Total	63

J. L. (CML)

	Serum B_{12} = 8.225 mμg./ml.	
	cpm − background	Per cent recovered
Whole plasma (20 ml.)	510	
Ppt. A	56	10.9
Ppt. B	156	30.6
Ppt. C	0	0
MP-1	197	38.6
	Total	80.1

DISCUSSION

It has been previously demonstrated that the phosphotungstic acid precipitate of a perchloric acid filtrate of plasma represents approximately 1.5 per cent of the total plasma protein and is rich in glycoproteins (14). This fraction is referred to as seromucoid (14).

Seromucoid is a heterogeneous material. Its major component was isolated in an electrophoretically homogeneous state by a series of ammonium sulfate precipitations by Weimer, Mehl and Winzler (15) and has been designated oroso-

mucoid or "MP-1." A second component has been demonstrated by electrophoresis and designated "M-2" (17). Experiments in progress suggest the presence of several further components (18).

The data presented in the present study suggest that the plasma B_{12} binding substance in both subjects with normal serum B_{12} concentrations and in CML patients with high serum B_{12} concentrations appears in the seromucoid fraction of plasma. That radioactivity represented the presence of B_{12} and not simply Co^{58} was demonstrated by the poor recovery of radioactivity in the seromucoid fraction when plasma was labeled with $Co^{58}Cl_2$. Because of the possibility that perchloric acid had stripped the $Co^{58}B_{12}$ from its original binding protein thus allowing it to bind secondarily to a glycoprotein, the perchloric acid precipitation was performed in the presence of a high concentration of nonradioactive B_{12}. This did not impair recovery of radioactivity in the phosphotungstic acid precipitate. The results of *in vivo* labeling further support the concept that the native substance is in the seromucoid fraction.

The addition of increasing amounts of $Co^{58}B_{12}$ brought out differences between normals and patients with CML when concentrations greater than 1 mμg. per ml. of plasma were reached. Thus, at 10 mμg. per ml. control plasma bound 16 per cent of added $Co^{58}B_{12}$, whereas plasma from CML patients with a high serum B_{12} bound a mean of 47 per cent. Studies by others (3, 4), also using dialysis methods, have demonstrated a comparable increase in the binding capacity for added B_{12} ("unsaturated binding capacity") in plasma from CML patients, though variations in the technique used probably account for differences in absolute values obtained by these authors.

With control plasma, when a concentration greater than approximately 2 mμg. of bound $Co^{58}B_{12}$ per ml. of plasma was reached, the per cent bound by the seromucoid fraction diminished whereas the per cent bound by the perchloric acid precipitate increased. This suggests relative saturation of the normal B_{12} binding substance at about the 2 mμg. per ml. level and subsequent secondary binding of additional B_{12} by perchloric acid precipitable protein. In two CML patients with high serum B_{12} concentrations, however, even at 10 mμg. of bound $Co^{58}B_{12}$ per ml. plasma, there was no decrease in the per cent bound to the seromucoid fraction.

More specific identification of the B_{12} binding material was attempted by using the scheme of Weimer, Mehl and Winzler (15) for the isolation of MP-1. These studies indicate that in sera from subjects with normal B_{12} concentrations the MP-1 fraction is not the major B_{12} binding substance. Instead, the bulk of radioactivity was found in Ppt. A (globulins) and could be separated from it by precipitation with perchloric and phosphotungstic acid. Plasma from CML patients, especially those with a high serum B_{12}, displayed a variable pattern with less activity noted in Ppt. A and more in Ppts. B, C and MP-1. The significance of this difference remains to be determined.

Though the present study clearly demonstrates that the plasma B_{12} binding substance appears in the seromucoid fraction of plasma, purification and chemical characterization of the material are required before one can say with certainty that it is a glycoprotein. Similar procedures are necessary to resolve the question of whether the increase in B_{12} binding substance seen in CML represents an excess of the normally occurring material or the presence of a chemically similar but abnormal material having B_{12} binding capacity. Studies on the further purification of the B_{12} binding protein by anion-exchange cellulose column chromatography (19) of the seromucoid fraction are now in progress (18).

The data presented appear to be in accord with the previously known facts concerning the plasma B_{12} binding substance. During electrophoresis at pH 8.6 in veronal, Pitney, Beard and Van Loon (1) found most of the endogenous bound B_{12} in the α-globulin fraction of serum as did Ostrowski, Skaryzynski and Zak (9) and Heinrich and Erdmann-Oehlecker (8). In a previous report from this laboratory (10), these findings were confirmed using paper or block electrophoresis followed by microbiologic assay of the endogenous B_{12} present in each fraction. It is known that under the same conditions of electrophoresis the bulk of the seromucoid fraction of plasma has a similar mobility (17). It is of interest that in all of these electrophoretic studies smaller amounts

of B_{12} were noted in the albumin and β-globulin fractions. It is not yet clear whether these fractions bind some B_{12}, since the spreading may be due to imperfections of methods.

The divergent results of Miller and Sullivan (4), who found that following the addition of 15 mμg. of $Co^{60}B_{12}$ per ml. of serum and paper electrophoresis at pH 8.6 in veronal the greatest radioactivity appeared in the β-globulin fraction in normals and in the α_1-fraction of sera from CML patients, may reflect the effects of *in vitro* "overloading" in the normal sera as a result of the unphysiologic concentration of added radioactive B_{12}.

Mendelsohn, Watkin, Horbett, and Fahey (10) have previously reported that when whole serum was fractionated by anion-exchange cellulose (DEAE) column chromatography a single B_{12} peak was obtained. The latter had the mobility of α_1-globulin whether obtained from normal or CML sera. On their chromatogram the B_{12}-containing fraction did occur in an area of relatively high protein-bound carbohydrate (hexose) concentration. It did not correspond with the site of elution of orosomucoid (MP-1) thus confirming the present finding that MP-1 is not the major B_{12} binding substance.

Recently, Miller and Sullivan have reported (20) that normal serum mucoprotein remaining after sulfosalicylic acid precipitation of proteins retained 13 per cent of total serum B_{12} binding capacity and CML mucoproteins so prepared retained 59 per cent. It is possible that the mucoprotein techniques used may impair the subsequent binding properties of the material. The present procedure of allowing B_{12} to bind to whole plasma and then fractionating appears to give better recovery of the B_{12} protein complex. The poorer recovery obtained by Miller and Sullivan may also be due to the use of sulfosalicylic rather than perchloric acid.

The fact that Gregory and Holdsworth (21) have described a B_{12} binding protein in sow's milk which has the characteristics of a glycoprotein and that a glycoprotein substance has been isolated from urine (22) which has strong B_{12} binding activity lends further support to the originally proposed hypothesis that various normally occurring B_{12} binding proteins are chemically similar. The subjects of the exact interrelationship between these substances as well as their physiologic and biochemical functions bear further investigation.

If, as data from the present studies imply, the elevated serum B_{12} levels seen in CML are associated with an increase of a specific B_{12} binding glycoprotein present in the seromucoid fraction, the finding is of interest in terms of the relation between neoplastic disease and glycoprotein metabolism. Total seromucoid as well as MP-1 and MP-2 are known to be elevated in the sera of human subjects with neoplastic disease (23) and also in certain tumor bearing animals (24). Total seromucoid is also known to rise in a variety of diseases associated with traumatic, inflammatory or degenerative tissue changes. These findings have been well reviewed by Winzler (23), Greenspan (25), and Moschides, Stefanini, Magalini and Kistner (26). In addition, however, there is evidence that the carbohydrate to protein ratio of seromucoid may vary in different diseases (27, 28) and also that the relationship between carbohydrate components themselves may vary (29). It has been previously suggested (26) that, since seromucoid is a heterogeneous material, these changes could result from the alteration in a specific component of this fraction. The present data suggest that such is the case in plasma of subjects with CML, in which the B_{12} binding protein is increased. The source of this material as well as the cause for its increase in this disease is at the present time unknown.

SUMMARY

The fact that intrinsic factor is thought to be a glycoprotein led us to investigate the B_{12} content of the seromucoid fraction of plasma from normal subjects and patients with chronic myelocytic leukemia (CML). When 0.1 to 1.0 mμg. of $Co^{58}B_{12}$ per ml. was added to plasma from six normal subjects and eight patients with CML and free B_{12} was removed by dialysis, 77 per cent and 83 per cent, respectively, of the bound radioactivity for the two groups was found in the seromucoid fraction. When 10 to 100 mμg. of $Co^{58}B_{12}$ per ml. was added to plasma from patients with CML, an association between the increased binding capacity of this plasma and the increased recovery of radioactivity in the seromucoid fraction

was demonstrated. The failure to recover substantial radioactivity from the "MP-1" (orosomucoid) fraction following its isolation by ammonium sulfate precipitation from the labeled plasma of normal subjects and patients with CML indicates that orosomucoid is not the major plasma B_{12} binding protein. The fact that the B_{12} binding protein is present in the seromucoid fraction of plasma suggests that it is a glycoprotein. In order to establish this, further purification and chemical analysis of the protein are required. These studies are now in progress.

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THE DEMONSTRATION OF PULMONARY ARTERIOVENOUS SHUNTS IN NORMAL HUMAN SUBJECTS, AND THEIR INCREASE IN CERTAIN DISEASE STATES *

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The evidence for existence of arteriovenous shunts in the normal human lung is strong but necessarily indirect. Simultaneous measurements of oxygen tension in alveolar air and in arterial blood consistently show a degree of venous admixture in both normal and abnormal subjects (1), but no information has been obtained about the path taken by this unventilated blood. The passage of small glass or plastic beads through the pulmonary circulation has demonstrated potential pathways as large as 200μ in diameter (2, 3), but this technique involves an artificial distention of the vascular bed and gives no information about the extent to which such pathways may function physiologically.

The present investigation was based on the hypothesis that the decreased venous inflow to the right atrium and reduced blood flow through the pulmonary vessels during the Valsalva maneuver, as well as the rebound effects after release of the maneuver, might alter the flow through such shunts, and that this alteration could be detected by continuous measurement of the systemic arterial oxygen saturation. No distinction would necessarily be made by this technique between true arteriovenous anastomoses, with ventilation-perfusion ratio of zero, and poorly ventilated alveolar capillaries, with ventilation-perfusion ratio below the average for the whole capillary bed.

We have studied nine normal subjects, and 16 patients with cardiac or pulmonary disease; results in 12 additional patients with intracardiac shunts have been included for comparison.

METHODS

Ear oximetry proved unsatisfactory in recording changes in saturation during the Valsalva maneuver, owing to the large variations in blood content of the ear caused by the maneuver. The Colson Model 103 cuvette densitometer [1] was therefore used, recording the optical density of arterial blood at 620 mμ wave length. As shown by Sabiston, Khouri and Gregg (4), in this portion of the spectrum the optical density of blood is a linear function of the per cent oxygen saturation of hemoglobin, provided no change in either hematocrit or velocity of blood flow through the cuvette occurs.

Patients were examined resting in the supine position. The cuvette densitometer was connected by approximately 20 cm. of polyethylene tubing (internal diameter = 1.14 mm.) to a needle inserted into the brachial or femoral artery. Arterial blood was drawn through the cuvette by a motor-driven syringe at a constant rate, usually 0.3 ml. per second. The output of the densitometer was recorded on one chanel of a Sanborn "Poly-Viso" or an "Electronics for Medicine" recorder. The sensitivity of the record was adjusted so that a 1 per cent change in oxygen saturation would produce a deflection of 1 to 2 mm., using the neutral density wedge of the Colson cuvette as a guide.

Calibration of the record for each patient was calculated after completion of the test, when his arterial hematocrit had been determined. Since the change in optical density of blood for a given change in oxygen saturation depends on hemoglobin concentration, a calibration chart could be prepared to give the sensitivity of our instrument at different hematocrit levels. The error introduced by using hematocrit as an indication of hemoglobin concentration (*i.e.*, assuming the same mean corpuscular hemoglobin concentration for all patients) is negligible for this purpose.

The calibration chart shown in Figure 1 was constructed from data obtained by *in vitro* measurements on four different human blood samples of widely different hematocrit values. In each sample the oxygen saturation was varied by aliquots of high and low saturation and the linear relation between optical density and oxygen saturation (4) determined. The slope of this line for each sample is plotted on the ordinate of Figure 1, and its hematocrit on the abscissa. Using this chart and a calibrating signal of known optical density on each record, the recorded deflections could be converted into changes in oxygen saturation. Figure 2 shows one of the original records, with the calculated sensitivity scale at-

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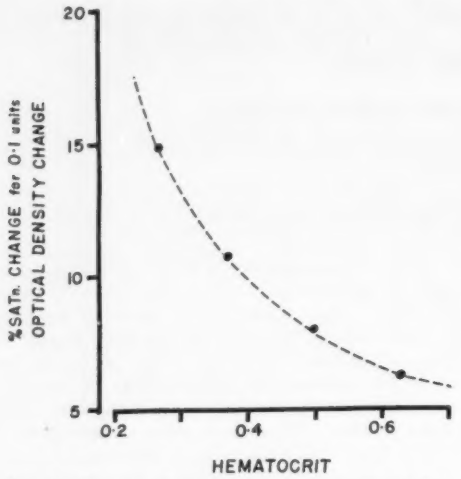

FIG. 1. RESULTS OBTAINED BY THE *IN VITRO* STUDY OF FOUR HUMAN BLOOD SAMPLES, SHOWING THE RELATIONSHIP BETWEEN HEMATOCRIT AND DENSITOMETER CALIBRATION

See text.

tached. To facilitate comparison, the records in Figure 3 have been replotted at uniform sensitivity.

A sample of arterial blood was taken immediately before each record and the control arterial oxygen saturation measured in a photometric oximeter (5) which was calibrated frequently with simultaneous Van Slyke determinations. Blood withdrawal through the cuvette was then begun, and after establishing a baseline on the record, the patient was asked to perform a previously rehearsed Valsalva maneuver, blowing through a mouth tube into a closed air chamber. A mercury manometer visible to the patient measured the pressure thus produced and the patient endeavored to keep this pressure at 40 mm. Hg for 12 to 14 seconds. In most cases, special care was taken to avoid a deep inspiration before the maneuver; when this was neglected, a deflection related to this inspiration frequently appeared in the record of arterial saturation, preceding that related to the maneuver itself, (Patients E.J. and M.G., Figure 3).

In each case the mouth pressure developed during the test was recorded by a strain gauge and amplifier to give an approximate indication of the changes in intrathoracic pressure (6) which constitute the primary effect of the Valsalva maneuver.

Pulmonary arterial pressure was recorded in three patients in whom the study was performed during right heart catheterization.

Baseline stability in the group of normal subjects was such that no measurable saturation changes occurred during quiet respiration. In the group with heart or lung disease, those whose condition was primarily cardiac showed an equally stable baseline; three patients, in each of whom the primary pathology was in the lungs, showed regular changes in saturation synchronous with respiration and of magnitude 0.4 to 0.5 per cent on either side of the mean. Saturation was recorded in six patients during simple breath-holding for 12 seconds; in four no saturation change was found, while in two there was a fall in saturation, of 0.6 per cent in one and of 1.1 per cent in the other. Three rapid deep inspirations in the same six patients caused no change in saturation in four, a rise of 1.2 per cent in one and a rise of 1.8 per cent in one. In no case was there any difficulty in distinguishing the effect of the Valsalva maneuver on arterial saturation from the effects of simple respiration. This was equally true in the patients with intracardiac shunts, although those with "balanced" shunts frequently showed respiratory fluctuations in saturation as large as 2 per cent on either side of the mean.

The changes in optical density of blood observed during and after the Valsalva maneuver could theoretically be due to acute changes in hematocrit rather than changes in oxygen saturation, although hematocrit

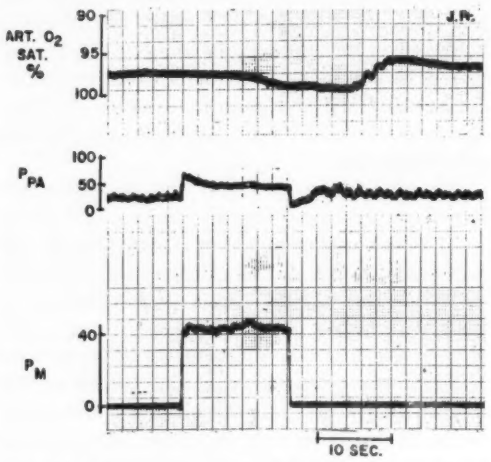

FIG. 2. ORIGINAL RECORD, SHOWING CHANGES IN BRACHIAL ARTERY OXYGEN SATURATION (ART. O_2 SAT.), PULMONARY ARTERY PRESSURE (P_{PA}, MM. HG) AND MOUTH PRESSURE (P_M, MM. HG.) DURING AND AFTER A VALSALVA MANEUVER IN PATIENT J.PR.

Effective pulmonary vascular perfusion pressure, estimated as the difference between mean pulmonary artery pressure and mouth pressure, falls from a control level of 24 mm. Hg to 6 mm. Hg within a few seconds after the onset of the Valsalva maneuver, maintains this level during the maneuver and rebounds temporarily to 38 mm. Hg after release. Increased arterial saturation follows the decreased perfusion pressure during the maneuver, and decreased saturation below control levels follows the rebound elevation of perfusion pressure. The time lag of approximately five seconds between pressure and saturation changes presumably represents transit time of blood from pulmonary capillaries to peripheral artery.

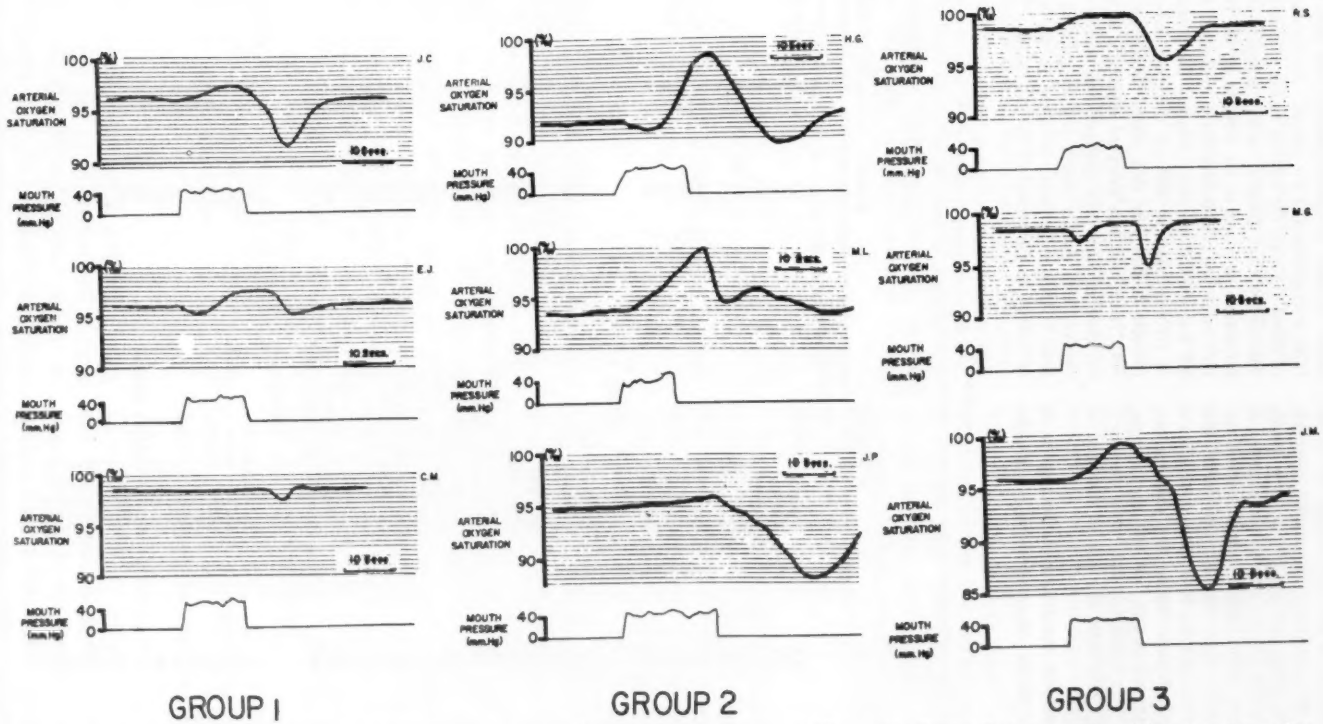

FIG. 3. RECORDS REPLOTTED AT UNIFORM SENSITIVITY, SHOWING THREE NORMAL SUBJECTS (GROUP 1), THREE PATIENTS WITH HEART OR LUNG DISEASE WHO SHOWED LARGE SATURATION CHANGES (GROUP 2) AND THREE PATIENTS WITH INTRACARDIAC SHUNTS (GROUP 3)

changes of the order of 10 per cent would be necessary. To rule out this possibility the studies were repeated in four patients using light of 800 mμ wave length, so that the record would not be affected by changes in oxygen saturation, but would reflect changes in hematocrit with a sensitivity approximately two-thirds that at 620 mμ. The records of these four patients at 620 mμ all showed significant changes during the Valsalva maneuver, larger than normal in two. At 800 mμ, however, three of the records were completely flat, while the fourth showed a very small deflection opposite in direction to that seen at 620 mμ.

Changes in flow rate of blood through the cuvette, if they were to occur during the maneuver, would also cause changes in optical density and lead to a similar false interpretation of the record. An increase in velocity of blood through the cuvette causes a diminution of the recorded optical density, owing probably to incomplete turbulence of blood in the cuvette at the rates of blood flow employed. This sensitivity to flow is equal at both 620 mμ and 800 mμ wave lengths; the absence of change in records taken at 800 mμ therefore indicates that no changes in flow rate took place. Moreover, the syringe withdrawal unit was designed especially to avoid the occurrence of such flow changes, and with experience, its operator can readily detect any irregularity in its function. Changes in blood pressure in the cuvette cause no change in the record of optical density.

TABLE I*

Patient	Age	Diagnosis	Arterial O_2 saturation		
			At rest	Rise during maneuver	Fall after maneuver
		Group 1			
			%	%	%
A. C.	18	Normal	97	2.5	None
D. B.	32	Normal	96	1.7	0.5
J. W.	40	Normal	99	0.8	0.7
L. D.	28	Normal	99	0.8	1.7
E. J.	58	Normal	96	0.9	1.3
J. C.	43	Normal	96	0.7	4.5
C. M.	31	Normal	98	None	0.9
D. L.	18	Normal	96	1.0	0.9
A. B.	32	Normal	95	1.1	2.6
		Group 2			
B. B.	47	CHF, (Isch.)	94	None	None
M. V.	58	CHF, MI, AI	85	None	None
A. Br.	27	MS	95	1.8	0.2
G. H.	36	MS, PH	98	1.2	1.0
J. Pr.	32	MS (mild)	98	1.9	1.5
B. Br.	45	Previous LVF (Isch.)	97	1.4	0.4
L. E.	56	Cirrhosis of liver	97	2.7	None
F. M.	61	Previous LVF (Isch.)	92	2.1	0.5
R. F.	48	Previous LVF (Isch.)	98	2.1	None
M. L.	46	Previous LVF (Isch.)	94	6.0	None
H. G.	58	PH, cause uncertain	92	6.4	2.0
H. A.	48	PH, lung fibrosis	85	7.3	None
A. Cu.	27	PH, lung disease	81	6.9	0.5
C. Y.	41	PH, cause uncertain	92	5.4	None
D. H.	54	Pulmonary emboli	94	3.9	3.3
J. P.	36	Previous cor pulmonale	93	0.5	7.2
		Group 3			
M. T.	25	ASD, Left-to-	95	2.4	None
R. S.	18	ASD right	95	1.6	1.5
M. G.	19	ASD shunt	99	0.8	4.4
G. W.	26	ASD	99	0.9	0.9
M. B.	34	VSD	98	1.2	0.4
E. W.	48	ASD, PH	92	5.5	7.9
I. N.	38	ASD, Ebstein's disease	70	0.4	4.7
D. G.	33	VSD, PS	90	None	10.7
J. S.	22	VSD, PH	86	0.9	7.8
J. R.	56	VSD, PH	87	2.7	8.2
J. B.	28	VSD, PH	94	3.6	16.6
J. M.	24	PDA, PH	96	3.5	13.3

* Key to abbreviations: CHF = congestive heart failure; Isch. = coronary artery disease; MI = mitral insufficiency; AI = aortic insufficiency; MS = mitral stenosis; PH = pulmonary hypertension; ASD = atrial septal defect; VSD = ventricular septal defect; PS = pulmonic stenosis; PDA = patent ductus arteriosus.

PATIENT SELECTION

Group 1 comprised two healthy members of the medical staff (C.M. and A.B.) and seven hospital patients who had no cardiovascular disease.

Group 2 patients all had obvious disease of the heart or lungs or both. Patients with severe emphysema were not included. The individual diagnoses are listed in Table I. The stated diagnosis was confirmed by autopsy in Patients M.L., A.Cu. and L.E., and at mitral valvotomy in A.Br. and G.H.; none of these five patients had a patent foramen ovale. Patients H.G., H.A., D.H. and J.Pr. were studied by cardiac catheterization and dye dilution techniques, and H.A. by angiocardiography; in none was there any evidence of an intracardiac shunt. The diagnosis in the remaining seven patients was made clinically. In J.P. the diagnosis of "Pickwickian syndrome" had been made one year prior to this study, after a complete hospital investigation. By strict dietary treatment, he had since lost over 100 pounds in weight and made a complete clinical recovery.

Group 3 included five patients with large left-to-right intracardiac shunts, and seven in whom the shunt was bidirectional (Table I).

RESULTS

The changes recorded in all the patients studied have been listed in Table I.

Group 1

A measurable change in saturation was recorded during or after the maneuver in every normal subject. The pattern of change was closely similar in each case; a rise in saturation occurred during the maneuver in eight of the nine subjects and a fall in saturation followed release of the maneuver in eight of the nine; both changes were seen in seven patients. The saturation rise averaged 1.2 per cent, varying from 0 to 2.5 per cent; the saturation fall averaged 1.5 per cent, varying from 0 to 4.5 per cent. The three records of normal subjects shown on the left of Figure 3 illustrate the greatest (J.C.) and least (C.M.) changes observed in normal subjects.

Group 2

The two patients who were in congestive heart failure at the time of study showed no change whatever in saturation during or after release of the maneuver; they were the only patients studied in whom no change was observed. In one of them (B.B.), a right atrial pressure record was made during the maneuver and showed the characteristic "square-wave" pattern found in patients with congestive heart failure (7).

In the other 14 patients of this group, the changes seen were of the same pattern as those in normal subjects, with a rise in saturation during the maneuver in all 14 and a fall in saturation after release in 12, reaching to below the resting level in nine. In seven of these 14 patients, the magnitude of the saturation changes was within the range seen in normal subjects, but in the remaining seven, changes of increased magnitude were recorded, rises of from 3.9 to 7.3 per cent occurring during the maneuver in six and a fall of 7.2 per cent following release in one. The records of three such patients, showing increased saturation changes, are illustrated in the center of Figure 3.

Group 3

The direction of saturation changes was again similar. Three of the four patients with predominant left-to-right shunt at atrial level showed changes differing in no way from those seen in normal subjects (*e.g.*, Patient R. S. in Figure 3); the fourth (M.G. in Figure 3), differed not in the magnitude but in the abruptness of the fall in saturation following release. The one patient with left-to-right shunt at ventricular level showed changes identical to those in normal subjects.

The seven patients in whom the shunt was "balanced" showed changes in the same direction, but of generally greater magnitude, as illustrated by the record of J.M. in Figure 3. In this group, respiratory fluctuations of plus or minus 2 per cent were common during quiet respiration and increased markedly to plus or minus 4 to 7 per cent on hyperventilation.

DISCUSSION

Patients without intracardiac shunts

The arterial saturation changes recorded in the 23 patients with no intracardiac shunt (Groups 1 and 2) presumably arose in the lungs. Functional patency of the foramen ovale might permit a small right-to-left flow of blood after release of the Valsalva maneuver, as does an atrial septal defect (8), but this would be expected in less

than 10 per cent of the adult population (9) and would not in any case explain the observed rise in saturation during the maneuver. Although a small amount of coronary venous blood is returned to the left side of the heart through the Thebesian vessels, this flow is probably too small to be of significance in the present results.

The intrapulmonary mechanisms which might account for our results may be divided into: changes in alveolar oxygen tension (alveolar pO_2), in diffusion capacity, in velocity of capillary blood flow or in distribution of blood flow within the pulmonary vascular bed. Our evidence and that of other investigators tend to exclude the first three of these factors.

Although we did not measure oxygen tensions, the calculated effect of the initial 40 mm. Hg rise in intrathoracic pressure is to raise a normal alveolar pO_2 (104 mm.) by 5 to 6 mm. at most; as the maneuver is held for 12 to 14 seconds, the pO_2 gradually falls, reaching close to the control value; when intrathoracic pressure is released, there is an immediate fall followed by a small rise caused by a brief period of hyperventilation. The whole range of alveolar pO_2 changes in normally ventilated alveoli probably does not exceed 5 to 6 mm., and this could not account for any measurable saturation changes in arterial blood. In poorly ventilated alveoli (*e.g.*, pO_2 50 mm.), the rise in pO_2 due to increased intrathoracic pressure would be proportionally less (at most 3 mm.) and, except with gross ventilation defect and marked arterial blood desaturation, this could not cause significant arterial saturation changes.

The Valsalva maneuver is unlikely to change gas distribution where this is already abnormal and we see no reason to suppose that the alveolar pO_2 in such an area would be elevated by any mechanism other than that discussed above. Even if it were to occur, such a change would be smaller than that caused by several deep inspirations, and as has been described, these never caused saturation changes comparable in magnitude to those accompanying the Valsalva maneuver.

An alteration in the diffusion properties of the alveolar membrane sufficient to account for the observed saturation changes seems improbable, because of the rapidity, the brief duration and the reversibility of the changes.

The absence of saturation changes in the two patients studied during congestive heart failure suggests that the changes are dependent upon the hemodynamic effects of the Valsalva maneuver, which are not obtained in such patients (7), rather than the ventilatory effects.

Simple changes in velocity of blood flow through pulmonary capillaries during and after the maneuver would not significantly alter the end-capillary oxygen content, as equilibrium between alveolar and capillary oxygen tension is achieved so rapidly (1, 10). The decreased pulmonary blood flow of systemic shock, for example, or the increased pulmonary flow in congenital left-to-right shunts, do not *per se* produce abnormal pulmonary venous oxygen saturation.

The explanation of our findings which we believe to be correct is that a redistribution of blood flow occurs within the pulmonary vascular bed during the maneuver. If in the resting state a small proportion of the pulmonary blood flow were distributed to unventilated shunt vessels, and if during the maneuver the flow through such vessels were diminished, the oxygen saturation of the mixed arterial blood would rise and the magnitude of the rise would be determined by the amount by which such shunt flow was reduced relative to the total flow. Similarly, an increase in the relative flow through the shunt pathways following release of the maneuver would cause a fall in the arterial saturation.

There is no direct evidence in the present results to indicate which particular feature of the hemodynamic responses to the Valsalva maneuver is responsible for the redistribution of flow. As described by Lee, Matthews and Sharpey-Schafer (11), both transmural pressure and flow in the pulmonary artery fall progressively during the maneuver and overshoot after release before returning to their resting levels. In Figure 2, the fall in transmural pressure correlates closely in pattern with the rise in arterial saturation, and the overshoot in pressure with the saturation fall. The same correlation between changes in transmural pulmonary artery pressure and in arterial saturation is found in patients with mitral stenosis on exercise (12) and on change of posture (13), in patients with atrial septal defect and pulmonary hypertension on exercise (14), and in dogs fol-

lowing injection of glass beads into the pulmonary artery (15). It is tentatively suggested therefore, that transmural pressure changes may influence patency of the intrapulmonary shunts, at least under the conditions studied here. Good evidence already exists that shunt vessels may be directly influenced by acetylcholine (16) and by the oxygen tension of inhaled gas (15).

All the six patients who showed saturation rises greater than the normal range had the clinical signs of pulmonary hypertension and this was confirmed by direct measurement in five (pulmonary artery systolic pressure above 50 mm. Hg). It is possible that the pulmonary hypertension itself may have been related etiologically to the presence in these patients of a large shunt flow. The Patient J. P. in Figure 3 (Group 2), who showed a fall of 7.2 per cent after release of the maneuver, may have had a patent foramen ovale. It is interesting, however, that he had suffered one year previously from an episode of cor pulmonale caused by obesity and hypoventilation; since then he had lost over 100 pounds in weight and had made a complete clinical recovery. An alternative explanation for the saturation fall is that it indicated the persistence of pulmonary shunt vessels developed during the earlier episode of cor pulmonale.

Uniformity of the hemodynamic response to the Valsalva maneuver must be assumed before the present findings can be compared quantitatively. Obvious exceptions occur in congestive heart failure, where little or no response is seen (7), and in emphysema, where greatly increased responses may occur (17). In the absence of emphysema, however, hemodynamic changes are rarely greater than normal and it is safe to assume that large saturation changes indicate large shunt flows.

The flow through shunt vessels at rest can be estimated from the rise in saturation and the cardiac output. In the group of normal subjects, the average rise of 1.2 per cent in saturation indicates a shunt flow at rest equal to about 1 per cent of the total pulmonary flow. In two of the six patients in whom a larger saturation rise was found, the cardiac output was measured and similar calculations indicated shunt flows at rest equal to 15 per cent and 20 per cent of the pulmonary flows.

The relation of the shunt vessels discussed to alveolar capillaries and to the bronchial circulation cannot be evaluated in the present study. If, however, the distribution of pulmonary blood flow between these vessels and ventilated alveolar capillaries is altered by simple changes in pressure or flow in the pulmonary artery, this would suggest that the two pathways differ anatomically and that the shunt vessel concerned is not, in fact, an unventilated capillary.

Patients with congenital cardiac shunts

In the patients of Group 3, the changes recorded in arterial saturation must represent redistribution of blood flow both within the lungs and across the intracardiac shunt, no distinction between the two being possible by the present method. Changes in the intracardiac shunt obviously predominated in some instances, producing much larger changes in saturation than were seen in patients of Groups 1 and 2. When the intracardiac shunt was predominantly from left to right, however, the saturation changes were often no larger than those seen in normal subjects. For this reason we cannot agree with the suggestion of Lee and Gimlette (8) that arterial saturation changes detected by the ear oximeter following release of the Valsalva maneuver are helpful in the diagnosis of atrial septal defect.

If pulmonary hypertension behaves in this group of patients as is suggested in those of Group 2, a large arteriovenous shunt may develop in the lungs. This intrapulmonary shunt may explain the pulmonary venous desaturation described in cases of atrial septal defect with pulmonary hypertension (14, 18), the arterial desaturation persisting after ligation of a patent ductus arteriosus complicated by pulmonary hypertension (19), and the presence of arterial desaturation in cases of ventricular septal defect with pulmonary hypertension insufficient to cause a reversal of the intracardiac shunt (20). This possibility should be considered before assuming that arterial desaturation indicates reversal of an intracardiac shunt.

SUMMARY

Arterial saturation changes have been recorded during the performance of the Valsalva maneuver

in 38 subjects, using a cuvette densitometer to give a direct and continuous record of oxygen saturation from arterial blood.

A consistent pattern of change in saturation was found in patients without intracardiac shunts, consisting of a rise in saturation during the maneuver and a fall in saturation following release of the maneuver. In nine normal subjects, the saturation rise averaged 1.2 per cent, while in six of the patients with cardiac or pulmonary disease, saturation rises of from 3.9 to 7.3 per cent occurred during the maneuver.

The most probable explanation for these findings is that the saturation rise results from a decrease in blood flow through pulmonary arteriovenous shunts, secondary to the hemodynamic effects of the Valsalva maneuver, and that those patients who showed a large rise in saturation had an abnormally large flow through such shunts at rest. The observations are consistent with the hypothesis that transmural pulmonary artery pressure is an important factor controlling flow through these shunts, and that an abnormally large shunt flow may develop in patients with chronic pulmonary hypertension.

Evidence of shunt flow at rest was found in eight of nine normal subjects and averaged approximately 1 per cent of total pulmonary blood flow.

In 14 patients with cardiac or pulmonary disease but without intracardiac shunts or signs of congestive heart failure, each case showed evidence of shunt flow at rest. The shunt was abnormally large in six of the 14, amounting to 20 per cent of total pulmonary flow in one subject. Two patients with congestive heart failure showed no change in saturation, presumably because the Valsalva maneuver produced no hemodynamic change in the pulmonary circulation.

The arterial saturation changes seen in 12 patients with congenital intracardiac shunts represented a summation of changes in shunt flow within the lungs and across the congenital defect, frequently dominated by the latter.

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APPLICATION OF CONTINUOUS FLOW ELECTROPHORESIS TO THE STUDY OF THE BLOOD COAGULATION PROTEINS. II. THE HEMOPHILIAS (A AND B) *

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A previous communication (1) concerned the application of continuous flow electrophoresis to the fractionation of normal blood preparations and the study of the coagulation proteins in these fractions. It was considered of some interest to apply the same techniques to pathological plasmas obtained from patients suffering from congenital deficiencies of AHF (antihemophilic factor A) and PTC (plasma thromboplastin component, Christmas factor, antihemophilic factor B). In addition, the isolation of an anti-AHF from the plasma of a hemophiliac with an acquired inhibitor is described.

MATERIALS AND METHODS

The methods have been described previously (1, 2). Plasmas were obtained from six patients, five of whom had suffered from severe and one (C.F.) from moderate hemorrhagic symptoms throughout their lives. Table I shows coagulation studies carried out on these patients on or near the date on which the blood for electrophoresis was obtained. Four patients suffered from AHF deficiency and two from PTC deficiency. All plasmas for electrophoresis were citrated except that of Patient R.R. This patient showed an acquired anti-AHF which we wished to isolate by electrophoresis. Thus, his plasma was prepared essentially free of other coagulation factors by employing sodium oxalate as anticoagulant, adsorbing with barium sulfate and heating to 56° C. for 10 minutes. This patient (R.R.) was interesting in that the appearance of anti-AHF could be shown to relate directly to transfusion of normal AHF. On his first three studies (age two and one-half) in January to June, 1956, no anti-AHF was demonstrable. In August, 1956, he received approximately 1,500 ml. of plasma over a five day period, and study three weeks later revealed anti-AHF titer of 1:64. During the next four months this gradually fell to zero and remained so for 17 months during which time he did not receive any blood or plasma. He was then transfused with 500 ml. fresh whole blood and tests six days later showed an anti-AHF titer of 1:64. It was at this time that blood for electrophoresis was obtained.

RESULTS

Hemophilia A. Figure 1 illustrates the results in one experiment (Patient T.M.) Paper strip electrophoretic patterns run on samples from each tube showed γ-globulin present in tubes 5 through 14, β in 15 through 17, α_2 in 18 and 19, α_1 in 21 and 22, α_1 plus albumin in 23 and albumin in 24 through 27. AHF activity was absent from the whole plasma and all fractions. Each fraction and the mother plasma were incubated with normal AHF and the residual AHF titer measured. No loss of activity was observed; thus no anti-AHF was present. The other coagulation proteins appeared normal in their distribution and concentration. Profibrinolysin and "glass factor" were in the γ area; heparin cofactor in the β area; prothrombin, proconvertin, PTC, antifibrinolysin and antithrombin in the α_1 to α_2 areas and proaccelerin in the albumin area. Quantities of substrate for measuring Hageman factor and PTA (plasma thromboplastin antecedent) were available for only a few tubes. These activities appeared primarily in the γ area.

The two other experiments on hemophilic plasma were very similar. The study on Patient B.T. showed no AHF or anti-AHF in the plasma or any fraction. The study on Patient C.F., a rather mild bleeder, showed some AHF activity (about 1.5 per cent of normal) in the whole plasma and in one fraction in the α_1 area.

Hemophilia A with anti-AHF. Anti-AHF activity (titer 1:2 to 1:8) was found in four tube fractions. The four tubes (10 through 13) were in the γ-globulin area and separate paper strip electrophoresis also showed these fractions to contain γ-globulin.

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TABLE I

Blood coagulation studies

Test*	Patient					
	B. T.	C. F.	T. M.	R. R.	J. P.	G. C.
Coag. time—glass (*min.*)	96	18	48	93	27	120
silicone (*min.*)	<300	90	<300	<240	<300	<240
Serum prot. time (*sec.*)	11.4	16.8	11.4	11.2	14	14.2
Prothrombin consumption (%)	0	17	0	0	10	14
Thromboplastin generation (%):	0	5	0	0	0	0
with normal $BaSO_4$ plasma	95	100	90	†	0	0
with normal serum	0	5	0	0	100	100
AHF assay (%)	0	1.5	0	0	60	100
PTC assay (%)	100	100	100	70	0	0
Anti-AHF	0	0	0	1:64		
Anti-PTC					0	0

* Serum prot. designates serum prothrombin; AHF, antihemophilic factor A; PTC, plasma thromboplastin component.

† Normal $BaSO_4$ plasma restored thromboplastin generation unless the patient's serum and the normal plasma were allowed to preincubate, in which case marked inhibition of the normal plasma was observed.

Hemophilia B. Figure 2 illustrates the results found in one experiment (Patient C.G.); the other was similar. Paper strip electrophoresis carried out on each tube showed Tubes 5 through 13 to contain γ-globulin; 14 through 16, β-globulin; 19 through 20, α_2-globulin; 21, α_1-globulin; 22 through 24, α_1 plus albumin and 25 through 29, albumin. PTC activity was absent from all fractions. No anti-PTC could be demonstrated in the whole plasma or the fractions. Profibrinolysin, glass factor, Hageman factor and fibrinogen were found primarily in the γ area; antithrombin, heparin cofactor, antifibrinolysin, proconvertin, prothrombin and AHF in the α_2 to α_1 areas and proaccelerin in the albumin areas. PTA activity was tested in three tubes and appeared in the γ area.

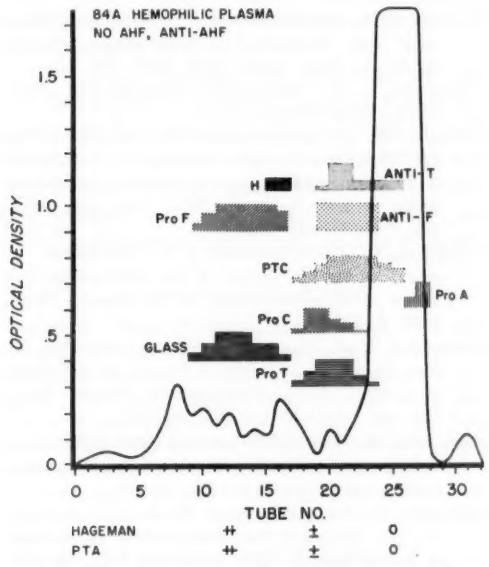

FIG. 1. CONTINUOUS FLOW ELECTROPHORESIS OF HEMOPHILIC PLASMA

The optical density curve indicates the relative protein concentration in each tube.

DISCUSSION

Most of the investigators in the field of blood coagulation feel that Hemophilia A and B are disorders due to genetically controlled deficiencies of two distinctly different plasma proteins. A divergent theory (3, 4) explains Hemophilia A as due to the binding of AHF by a circulating inhibitor. If such an AHF-anti-AHF complex exists in hemophilic blood, it is not dissociated by electrophoresis as neither AHF nor anti-AHF activity could be found in any of the fractions from the plasmas of the severe hemophiliacs. In the study on the mild hemophiliac a trace of AHF was found in the same location (α_1) in which it appears in normal plasma.

The inhibitor to AHF, demonstrated in the blood of Patient R.R., is probably not the same as that assumed to exist in the blood of all hemophilic patients by the exponents of the "inhibitor" theory of hemophilia. On two occasions it was possible to show a marked increase in the titer of this inhibitor after transfusions, followed by gradual diminution until no inhibitory effects on nor-

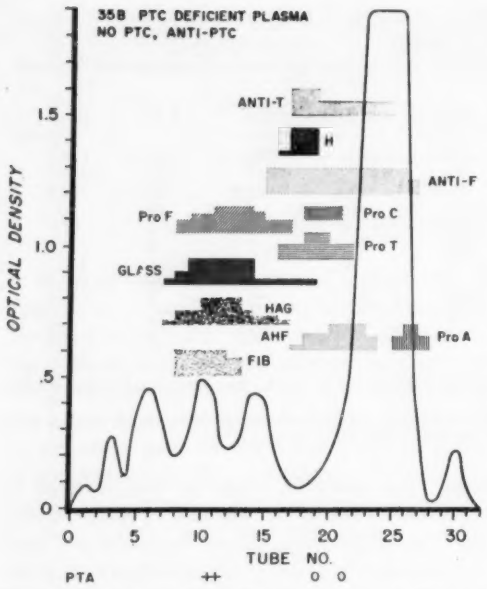

FIG. 2. CONTINUOUS FLOW ELECTROPHORESIS OF PLASMA THROMBOPLASTIN COMPONENT (PTC) DEFICIENT PLASMA

mal AHF could be demonstrated. In spite of these variations in inhibitor strength, AHF activity was never demonstrable. The inhibitory activity from the plasma of Patient R.R. was isolated in the γ-globulin fraction, confirming the previous findings of Craddock and Lawrence (5).

The abilities to correct the clotting "defects" in Hageman factor deficient plasma, normal silicone plasma ("glass factor") and PTA deficient plasma were present in the fractions from both Hemophilia A and B. The exact abnormality present in PTA plasma had not been completely defined. Rosenthal, Dreskin and Rosenthal (6, 7), who first described this disorder, felt that it was due to a deficiency of a unique clotting factor which they entitled PTA (plasma thromboplastin antecedent). On the other hand, Johnson, McClaughry and Seegers (8) described double deficiencies of platelet cofactors I and II (AHF and PTC) in PTA plasma. Our experiments suggest that both AHF and PTC deficient plasmas and certain of their fractions have the ability to correct the coagulation defect in PTA deficient plasma and, conversely, that plasma from a PTA deficient subject contains normal concentrations of AHF and PTC (9). The close similarity between the distribution of PTA, Hageman factor and "glass factor" in these and in normal plasma is being further explored.

SUMMARY

Continuous flow electrophoretic fractionation was applied to four AHF and two PTC deficient plasmas. One AHF deficient plasma showed the presence of anti-AHF. Fractions from the three ordinary hemophilic plasmas showed no anti-AHF and no AHF activity with the exception of one fraction from a mild hemophiliac which showed a trace of AHF activity. Fractions from the PTC deficient plasmas showed no PTC or anti-PTC activity. Other coagulation factors appeared normal in concentration and distribution. The anti-AHF activity of the plasma from the patient with an acquired inhibitor was concentrated in the γ-globulin area.

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TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. V. SUBFRACTIONATION OF THE ULTRAFILTRATE (UF–O) FRACTION *

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A procedure has been reported (1) for the reproducible separation of the total nondialyzable solids (TNDS) of normal human urine into three primary fractions. The most abundant of the three fractions, designated UF–O, was separated from the other two by virtue of its passage through a collodion ultrafilter membrane. The overall chemical composition of fraction UF–O was described, but no further information was then available. Accordingly, we have devised a procedure for the separation of this material into subfractions. While these subfractions appear to be cleanly separated, they are all probably mixtures of various chemical compounds. The method yields consistent results and therefore seems to be a useful tool for the study of possible changes in physiological and pathological conditions, and as an intermediate step in the ultimate separation of the components of UF–O.

This report describes the procedure for the subfractionation of the UF–O and the results obtained with it, including the range of normal daily variation in the excretion of the subfractions and their overall chemical composition. Some pertinent ancillary information about the TNDS, acquired during these experiments, is also included.

MATERIALS AND METHODS

Subjects. Three 24 hour urine specimens were collected, with chloroform preservative, by each of 12 normally active, healthy subjects (eight male, four female). With one exception, they were the same subjects as in preceding reports of this series (1–3). Two male subjects were Negroes. The collections were made during the period September 15 to December 10.

As each day's specimen was completed, the process of dialysis and subsequent fractionation of the TNDS was begun, as previously described (1, 2).

Fractionation procedure. The subfractionation procedure for fraction UF–O depends essentially on three observations. First, it had been noted (1) that the process of lyophilization effected a change in UF–O, manifested by the conversion of a large proportion of it to a dialyzable form. Preliminary experiments indicated that this was not the result of a purely random breakdown; instead, it seemed to be confined to some particular part of the UF–O. A further experiment showed that the lyophilization process alone, and not the fractionation *per se*, was responsible for this dialyzability, since concentration of UF–O by rotary evaporation did not cause any portion of it to become dialyzable.

The second observation was that, of the portion which did not become dialyzable after lyophilization, part was precipitable by saturated basic lead acetate, by 5 per cent phosphotungstic acid in 2 N HCl, or by saturation with ammonium sulfate. These respective precipitates were, of course, not necessarily of the same constitution in each case. Trichloracetic, perchloric, tannic or picric acids gave no precipitate either with UF–O or with this material. Ammonium sulfate was chosen as the most convenient and innocuous precipitant for use in further study.

Thirdly, part of the dialyzable portion of UF–O could be precipitated with either alcohol or acetone, the latter being chosen for routine use.

The details of the procedure that was finally adopted were as follows. The lyophilized UF–O material for each subject, representing the three day urine collection, was mixed with 25 ml. of distilled water, placed in a Visking® cellophane dialysis sac (0.75 inch inflated diameter, 1.0625 inch flat width),[1] leaving room for an increase in volume, and dialyzed 18 to 22 hours against 170 ml. cold distilled water. The dialysate, designated UF–1, was removed and recovered as a brittle, somewhat hygroscopic, yellow powder on lyophilization. Dialysis against several changes of cold water was continued for a further four days. This dialysate was discarded because of its impracticably large volume.

After dialysis, the dialysand was made to pH 4.0 with acetic acid and centrifuged.[2] The small, but very insoluble, centrifugate was washed and the washings added to the original supernate. The centrifugate was recovered on lyophilization as a gray or tan, nonhygroscopic, fluffy powder, designated UF–2.

* Supported by Mary Reynolds Babcock Foundation and Public Health Service Grant A-259.

[1] The experiments of Craig, King and Stracher (4) indicate that the choice of tubing would affect the results, since dialyzability decreases with increasing tubing size.

[2] All centrifugations were at 1,000 × G for 20 minutes.

The supernatant, at room temperature, had an excess of ammonium sulfate added. The saturated solution was centrifuged or filtered[3] after standing about one-half hour and the precipitate was washed with saturated aqueous ammonium sulfate; the washings were added to the supernate. The precipitate, designated UF–3, and the supernate, designated UF–4, were then separately dialyzed against cold water until free of ionic sulfate. Room for change of volume was allowed, so the cellophane would not be streched. UF–3 was a tan to dark brown, fluffy, nonhygroscopic powder on lyophilization; UF–4 was a cream-colored, fluffy, nonhygroscopic powder.

After weighing UF–1, 5 ml. of water was added and mixed. A small and very insoluble portion, designated UF–1A, was removed by centrifugation and washed with water. UF–1A was a pale gray or tan, nonhygroscopic powder on lyophilization. Five volumes of acetone were added to the supernate at room temperature. The resulting precipitate, designated UF–1B, was removed by centrifugation and washed with acetone. The soluble portion from the acetone precipitation, designated UF–1C, was checked for completeness of precipitation by further addition of acetone. The acetone was removed at 40 to 50° C., by means of a rotary evaporator. The residue was finally lyophilized to a yellow-brown syrup or a highly hygroscopic tar.

A flow sheet summarizing the above procedure is presented in Figure 1.

The figures in Tables II and III for "number of analyses" represent analyses of material from that many different individuals, insofar as possible. Where necessary, pools of material from three to five individuals were used for analysis.

[3] Eaton-Dikeman 42 mm. paper, No. 5268.

Analytical procedures. The methods used here have been previously described (1–3), except for the following additions.

A more convenient and equally accurate micromethod (5) was substituted in the nitrogen determination; a precision of 0.5 per cent or better was generally obtained among triplicate samples. The digestion was continued for one hour after the samples cleared.

Sialic acid was measured as before, but also by a more recently described procedure (6), except that *n*-octyl alcohol was substituted for amyl alcohol in the extraction and the final color was measured at 595 mμ.

A method for the estimation of those 6-desoxyhexoses (methylpentoses) present in UF–O was developed by modifying the usual anthrone procedure (7). It was observed that rhamnose and fucose developed their maximum color intensity with anthrone in a practically parallel fashion but faster, at a lower temperature, and have a somewhat different absorption maximum than did the hexoses present in UF–O. The color development of galactose and of fucose under the usual conditions and also under the conditions described below is illustrated in Figure 2, and is typical of the other hexoses (glucose, mannose) and 6-desoxyhexoses (rhamnose) in UF–O. The procedure was as follows. Solutions having 12.5, 25.0 and 50 μg. of rhamnose and of fucose per ml. were the standards. Two ml. of cold standard and 4.0 ml. of freshly prepared, cold anthrone reagent (200 mg. anthrone in 100 ml. sulfuric acid) were carefully mixed in a bath of crushed ice. The initial optical density, as compared with a water blank, was less than 0.04. Duplicate samples were similarly mixed. The tubes were allowed to come to room temperature, then placed in a water bath at 60° for exactly six minutes, rapidly cooled in crushed ice, again al-

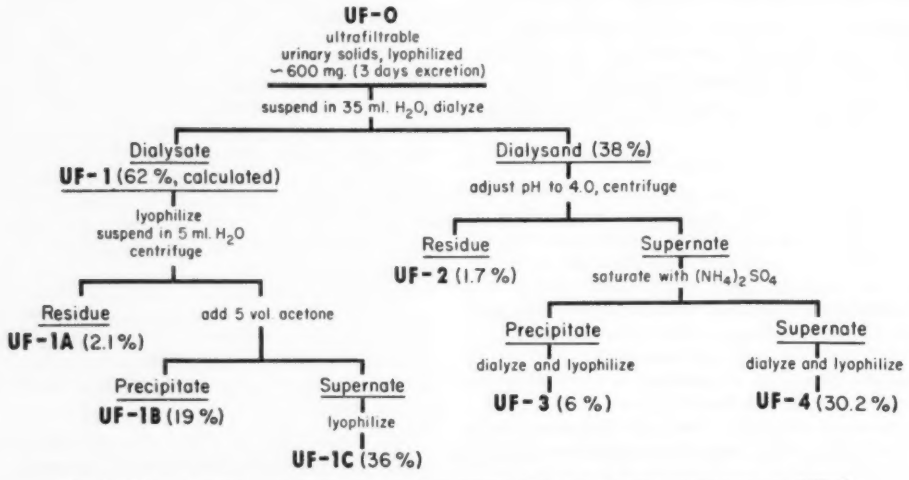

FIG. 1. FLOW SHEET SUMMARIZING THE PROCEDURE FOR THE SUBFRACTIONATION OF UF–O
Percentages represent average per cent of lyophilized UF–O found in each subfraction.

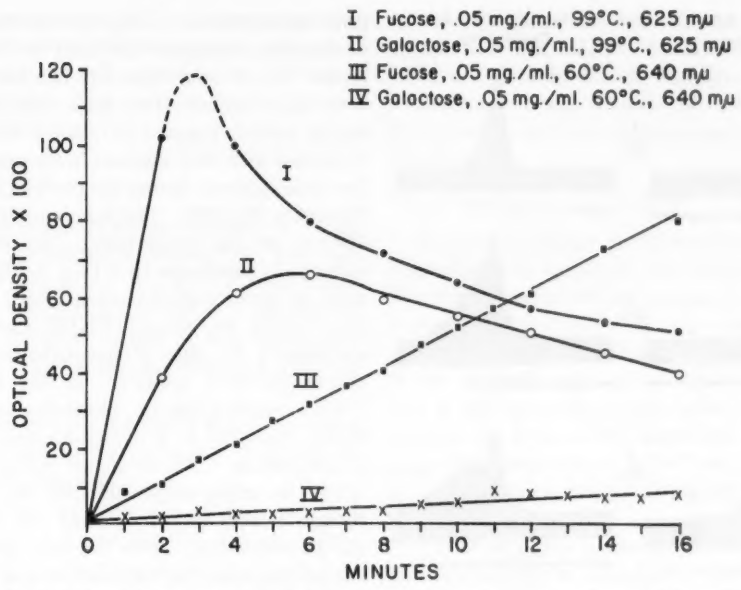

FIG. 2. COLOR DEVELOPMENT OF GALACTOSE AND FUCOSE WITH ANTHRONE REAGENT UNDER TWO SETS OF CONDITIONS

lowed to come to room temperature, and read vs. a water-reagent blank at 640 mμ. Under these conditions, the hexoses develop only about 10 to 15 per cent as much color as do the 6-desoxyhexoses. With respect to the present application, at least, the foregoing procedure is subject to much less interference than the cysteine-sulfuric acid reaction popularly used.

Electrophoresis was done as previously described (8), using a Spinco Model H electrophoresis-diffusion instrument.

RESULTS

Excretion rates

It may be seen (Table I) that the daily excretion of each of the fractions of UF–O is fairly constant for normal subjects. There were no apparent significant differences between older and younger, male and female, or white and Negro subjects. One subject, who had a common cold during the collection, showed a relatively increased UF–O (856 mg. per day). The increase was confined to the UF–1 material.

While only a portion of UF–1 was routinely recovered for each subject, experiments showed the analytical results obtained with this part to be representative of the total dialyzable material. An arithmetic extrapolation to the whole is therefore analytically valid. The portion of UF–1 actually recovered varied from 18 to 40 per cent (average 30.2 ± 7.5) [4] of the dialyzable part of UF–O,

[4] Plus or minus values in the text are standard deviations.

TABLE I

Average daily excretion of subfractions of ultrafiltrable fraction (UF–O) of TNDS by 12 subjects, in milligrams lyophilized dry weight*

Fraction	UF–O	UF–1A†	UF–1B†	UF–1C†	UF–2	UF–3	UF–4
Mean	196.8	2.6‡	38.2	73.7‡	3.4	11.9	59.5
Range	130–285	2.14–3.42	17.2–63.3	70.2–77.5	1.0–13.1	8.8–17.7	39.8–79.7
± S.D.	50.9		14.1		3.33	2.51	13.21

* Total nondialyzable solids.
† Values corrected for total (see text).
‡ Three pooled samples, each from three to five subjects.

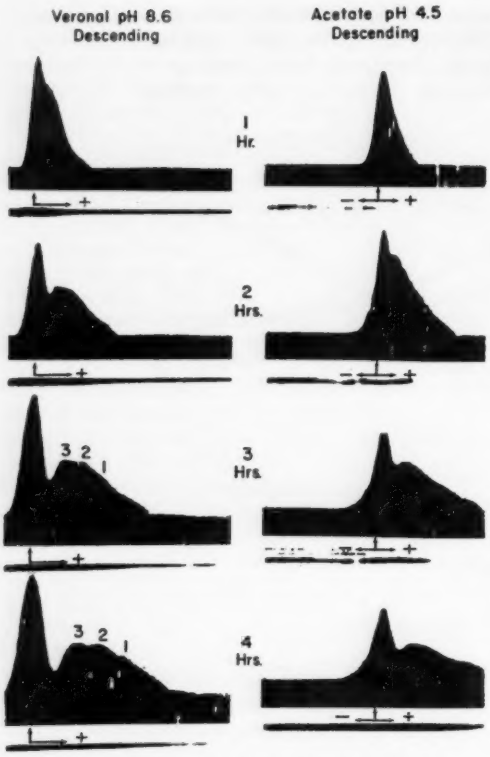

FIG. 3. CONCENTRATION INDICATED GRADIENTS OF FRACTION UF–4

Veronal buffer: ionic strength 0.1, pH 8.6, electropotential 6.4 volt/cm. Mobility of more clearly defined gradients 1, 2 and 3 were, respectively, -4.22, -2.98 and -2.04×10^{-5} cm.^{2}/volt sec. Acetate buffer: ionic strength 0.1, pH 4.5, electropotential 2.1 volt/cm.

which in turn comprised 52 to 73 per cent (average 62 ± 5) of the UF–O as calculated by the difference between the starting weight and the sum of the combined weights of the nondialyzable fractions.

Seasonal effect

The other two primary fractions of TNDS, designated R–1 and RS–1 (1), were also measured during this study. Within experimental error, they gave the same values as have been obtained on other occasions and during other seasons of the year, respectively, 82.9 ($\pm$ 29.4) and 51.3 ($\pm$ 20.7) mg. per day. However, the average value for UF–O (Table I) was lower than that found in previous experiments, although the standard error of the mean was about the same as before. Data on the UF–O from three day specimens collected from eight subjects (four male, four female) during the period August 1 to October 20, 1957, were compared with data obtained in the same way from the same subjects during the period October 11 to December 10, 1958. The values averaged 258 and 204 mg. per day, respectively. This difference was statistically significant ($p < 0.01 > 0.001$). Moreover, on plotting all data accumulated over a three year period on normal TNDS excretion (113 specimens) vs. date of measurement, there appeared to be a minimum in the excretion of TNDS reached during December and January, rising gradually to a maximum near the middle of September. The evidence indicates that the UF–O is solely responsible for this change, although a more thorough study will be necessary for quantification. From the data given below, it can be computed that this alteration in the quantity of UF–O reflects an average decrease of approximately 20, 14, 16 and 17 mg. per day in the respective excretion rates of hexose, sialic acid, protein and ash, as compared with the previously presented data (1). Which of the subfractions is responsible for this change has not yet been determined.

Precipitation experiments

The pH of maximum precipitation of UF–3 was 2.7 as determined by a series of increments of hydrochloric acid to a solution at pH 8.6. UF–4 gave no precipitate over the pH range 2.3 to 10.5.

Electrophoresis

Only one of the fractions, UF–4, was suitable for electrophoresis by the moving boundary technique. Figure 3 illustrates typical patterns obtained with this material, which look much like patterns obtained with lyophilized UF–O.

Serological tests

Of the mammalian mucosubstances known at present, fraction UF–4 is most nearly like the blood group substances in analysis (9). A 20 per cent solution of UF–4 was mixed with human anti-A and anti-B serum. A and B human red

blood cells were mixed with the corresponding antibody and suspected blood group substance. No decrease in red cell agglutination was noted as compared with anti-A and anti-B serum mixed with red blood cells.

Analytical results

The analytical results are summarized in Tables II and III. No sulfhydryl sulfur (*i.e.*, less than 0.1 per cent) was found in UF–O. Total sulfur (Carius procedure) was 1.25 and 2.27 per cent in two samples analyzed. Most of this was sulfate sulfur, but a quantitative measurement was not made.

Hexosamine was not detectable in UF–O before hydrolysis, indicating that none of it is present as a nonacetylated end group.

A measure of the adequacy of the analytical data was obtained by adding the average values (Tables II and III) for the percentage of each constituent in each fraction. The two values obtained for hexose and for sialic acid, respectively, were first averaged; the nitrogen was corrected for the nitrogen present as hexosamine and sialic acid and then multiplied by the factor 6.25 as an estimate of the peptide. The following percentages resulted: UF–1A, 93.7; UF–1B, 104.4; UF–1C, 97.2; UF–2, 128; UF–3, 96.9 and UF–4, 99.2. Fraction UF–2 was analytically erratic from sample to sample and had no consistently reproducible composition.

DISCUSSION

At present, almost no information is available concerning the origin of the foregoing fractions, how many components are present in each of them, or their natural chemical state in urine.

The value of the subfractionation is analogous to that of a controlled partial hydrolysis of a protein, in that some less complex parts of the starting material are reproducibly separated. Information about these segments of UF–O can, perhaps, now be obtained more easily and reliably, and eventually interrelated.

It is obvious that lyophilization has caused some drastic alteration in some part of the UF–O, since it has become dialyzable. Whether this represents the partial disintegration of a component or components of UF–O through rupture of primary bonds, the release of a moiety or moieties held by secondary bonds, or both, is unknown.

TABLE II

Average glucide composition of subfractions of ultrafiltrable fraction (UF–O) of TNDS, expressed as per cent of lyophilized dry weight

Fraction	Hexuronic acid		Hexosamine*	Hexose			6-Desoxy-hexose	Sialic acid	
	Naphth.	Carb.		Anthrone	Orcinol	Anth./Orc.		Diphenyl-amine	Resorcinol
UF–1A	0.25		0.0	3.0	2.4	1.25		0.0	0.0
n†	(2)		(2)	(2)	(2)			(2)	(2)
UF–1B	2.05	2.88	2.51	7.56	6.71	1.13	1.98	19.25	12.29
n	(9)	(5)	(8)	(9)	(9)		(6)	(9)	(9)
S.E. mean	0.25	0.45	0.23	0.67	0.61		0.04	2.29	1.86
UF–1C	1.84	4.47	15.0	5.2	6.6	0.79		2.47	1
n	(2)	(3)	(2)	(2)	(2)			(2)	(2)
UF–2	0.26		2.64	18.3	8.51	2.15		0.0	0.0
n	(4)		(4)	(2)	(2)			(4)	(4)
S.E. mean	0.01		0.52						
UF–3	0.36	1.15	8.26	17.17	19.32	0.92	4.95	0.0	0.0
n	(12)	(1)	(12)	(11)	(11)		(5)	(11)	(5)
S.E. mean	0.02		0.30	0.84	1.32		0.18		
UF–4	0.38	4.28	20.63	30.78	21.89	1.41	7.69	8.45	2.54
n	(12)	(4)	(11)	(11)	(11)		(7)	(11)	(7)
S.E. mean	0.02	0.38	0.57	0.90	0.80		0.43	1.66	0.45

* Expressed as glucosamine hydrochloride.
† n = Number of analyses.

Glucides

The major fractions of UF–O have a glucide content which is generally high and, in the case of fractions UF–3 and UF–4, is remarkably high. The results show that, in those fractions examined, one-third to one-fourth of the hexoses present are 6-desoxyhexoses. Preliminary paper chromatography after resin hydrolysis thus far has indicated UF–1B and UF–1C to contain (in order of decreasing intensities of spot colors) galactose and rhamnose; UF–4 contained galactose, fucose and mannose. The apparent proportions of these sugars is consistent with the anthrone/orcinol ratios of the respective fractions (1).

The resorcinol method (6) for determining sialic acid gave results consistently lower than did the diphenylamine method. We are not prepared to discuss the reason for this difference, which has been observed also with other sialic acid-containing materials (10). It should be noted, however, that one of the two absorption maxima in the region 450 to 900 mμ obtained by the diphenylamine method varies considerably in intensity from batch to batch of crystalline sialic acid standard. This is the maximum which may variably be found at 700 to 725 mμ. The reason for and nature of this variation, which may also occur in a long-refrigerated solution, is not completely clear. The other maximum, at 525 mμ, is the wave length at which measurements are routinely made, and has not varied significantly in wave length or extinction from batch to batch of standard. In UF–4, the absorption of the 700 mμ maximum is relatively so large that the final color is visually violet-blue instead of violet and consequently so broad that it must also considerably increase the absorption at 525 mμ, creating a positive error in the estimation.

The interpretation of the results for hexuronic acid is clarified by the following observations. The hydrolytic conditions for the original naphthoresorcinol method did not sufficiently hydrolyze a commercial sample of chondroitin sulfate (1 mg. per ml.) for a color reaction to be obtained with naphthoresorcinol (1). When the resin hydrolysis was tried instead, there was a partial but insufficient hydrolysis of chondroitin sulfate for accurate naphthoresorcinol determination.[5] A pre-

[5] A resin-hydrolyzed sample of commercial chondroitin sulfate containing 61 μg. hexuronic acid gave a pink, rather than a purple naphthoresorcinol reaction product, having a maximum at 517 mμ and equivalent in optical density (at 565 mμ) to 56 per cent of the theoretical value.

TABLE III

Average inorganic and nitrogen content of subfractions of ultrafiltrable fraction (UF–O) of TNDS, expressed as per cent of lyophilized dry weight

Fraction	Nitrogen	"Bound" water	Ash	Ca*	P*
UF–1A	0.34†	15.1	66.5	17.4	10.1
n‡	(2)	(2)	(2)	(2)	(2)
UF–1B	5.41	8.96	40.16	12.36	3.12
n	(9)	(5)	(5)	(5)	(5)
S.E. mean	0.41	0.49	4.19	1.41	0.74
UF–1C	4.83	16.13	16.52	2.42	0.90
n	(3)	(3)	(3)	(3)	(3)
UF–2	5.98	13.7	62.1	13.00	8.4
n	(4)	(2)	(3)	(3)	(2)
S.E. mean	1.40				
UF–3	9.11	11.55	6.13	26.35	8.1
n	(12)	(2)	(3)	(2)	(2)
S.E. mean	0.42				
UF–4	5.67	12.11	7.00	21.18	6.18
n	(12)	(13)	(12)	(12)	(12)
S.E. mean	0.39	1.46	0.63	1.77	0.62

* Per cent of ash.

† Separate samples gave 0.32 and 0.35 per cent N. Because of high ash content, a third sample was run by Dumas procedure and gave 1.50 per cent N.

‡ n = Number of analyses.

liminary hydrolysis is not necessary for determining hexuronic acids with carbazole, but extraneous color interference is a serious problem, which is much reduced by resin hydrolysis. In general, the differences between the results obtained (Table I) with carbazole (and resin hydrolysis) and with naphthoresorcinol (and acid hydrolysis) appear to be primarily, if not completely, a consequence of positive hexose or other interference, which is incompletely corrected for by a no-carbazole blank. Thus, the fractions generally contain only "non-acid mucopolysaccharide hexuronic acid." [6]

Fraction UF–4 (and possibly UF–3, the ultimate distinction being a matter of judgment) is an exception; the differences obtained with the two methods appear too large relative to the interference to be entirely accounted for in this way. Moreover, a strongly carbazole-positive material, with very little interference, can be precipitated from this fraction with cetyltrimethylammonium bromide (11); carbazole positive material, together with considerable interfering material, can also be isolated by the apparently less selective glacial acetic acid precipitation followed by ethanol precipitation from solution in 3 per cent potassium hydroxide. These same solubility properties have, of course, been used in the isolation of chondroitin sulfate.

In fraction UF–4, the result with resin hydrolysis and carbazole is therefore probably the most nearly accurate figure for "total hexuronic acid," while the acid hydrolysis and naphthoresorcinol method certainly provides the most nearly accurate figure for "'non-acid mucopolysaccharide hexuronic acid," and indeed approximates the daily excretion rate reported for such material (11, 12), the remainder being present in the RS–1 fraction (1), which contains a relatively small amount of bound hexuronic acid in each of the two forms.

Peptides

It is noteworthy that fraction UF–1B was strongly ninhydrin positive (three minute boiling) for samples from some individuals, but negative for others. Fraction UF–1C was negative, or only very weakly positive, in all. A positive ninhydrin test is generally indicative of free amino acids, hexosamine or short, simple peptides.

Inorganic constituents

In preceding reports (1, 2), it was stated that tests for phosphorus were negative prior to ashing. This observation was incorrect. A color sufficient to account for approximately all the phosphorus (as inorganic phosphate) is developed by the lyophilized, unashed UF–O.

The variations in content of ash, Ca and P among the fractions is noteworthy. It must be remembered that dialysis is an asymptotic process, however, and the most reasonable present explanation for the high ash contents of fractions UF–1A and UF–2 is that this represents some last traces of originally dialyzable (and reversibly bound) salts, which precipitated irreversibly on lyophilic concentration. The analytical inconsistency of UF–2 suggests that the organic portion probably was not originally bound to any considerable amount of this ash, but was present adventitiously as denatured material and/or traces of fraction R–1 (1) which managed to pass the ultrafilter membrane.

[6] By "acid mucopolysaccharide" is intended chondroitin sulfate(s) (or chondromucoprotein), heparin and/or hyaluronic acid.

SUMMARY

A method has been described for the separation of the ultrafiltrable fraction of the total nondialyzable solids of normal urine into six arbitrary but reproducible subfractions. The 24 hour excretion rates and the overall chemical composition of these six subfractions are presented.

ACKNOWLEDGMENT

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PLASMA CONCENTRATIONS OF EPINEPHRINE AND NOREPINEPHRINE DURING INTRAVENOUS INFUSIONS IN MAN * †

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Although numerous clinical studies have centered about the infusion of epinephrine and norepinephrine (1–3), the plasma concentrations attained by the infused catecholamines during such investigations have never been delineated. Recently a highly specific and sensitive procedure for the simultaneous fluorometric determination of epinephrine and norepinephrine in plasma was described (4, 5). With this procedure, a detailed investigation of plasma epinephrine and norepinephrine levels during intravenous infusions in man was undertaken and is now documented in the present communication.

EXPERIMENTAL

Infusion studies were performed on a group of volunteers, *viz.*, medical studients, and on a group of schizophrenic patients at the New York State Psychiatric Institute. Sterile saline solutions of *l*-epinephrine or *l*-norepinephrine (Suprarenin® bitartrate or Levophed® bitartrate monohydrate, Winthrop Stearns Inc., N. Y.) for infusion were prepared at concentrations of 0.5 or 4.0 μg. free catecholamine per ml. Each study was initiated with a 10 minute control period consisting of a saline infusion, following which the administration of epinephrine or norepinephrine was begun. Infusions were shifted at will from saline to catecholamine by means of a three-way stopcock. Flow rates were controlled by an infusion pump (Process and Instruments Co., New York) which permitted the instantaneous shifting of infusion speeds while the pump was in operation. Flow rates of 1.5 to 7.5 ml. per minute were employed.

A total of 41 infusions was performed; these included 24 infusions with 16 normal subjects (15 males and one female) between the ages of 21 and 29 years, and 17 infusions with 14 schizophrenic patients (six males and eight females) between the ages of 18 and 33 years. Subjects received infusions in the left antecubital vein while they were lying on a bed. Blood pressure and pulse were noted at various intervals. Routinely, 30 ml. blood specimens were drawn from the right antecubital vein into syringes wetted with heparin solution (sterile Liquaemin® Sodium, Organon Inc., Orange, N. J.), and then transferred immediately to cold 40 ml. centrifuge tubes equipped with ground glass stoppers. Specimens were chilled in an ice-water bath until the complete set for the particular experiment had been collected. They were then centrifuged at 700 G for 15 minutes following which the supernatant plasmas were removed and analyzed for epinephrine and norepinephrine as described elsewhere (4, 5). The plasma concentrations of catecholamine reported in this communication are uncorrected for recoveries which had been demonstrated previously to range from 70 to 90 per cent (5).

Infusions were automatically terminated after infusion speeds of 30 μg. catecholamine per minute had been reached, or if a pulse pressure of 100 mm. Hg or a basal pressure increase of 100 mm. Hg were attained. Cessation of infusions also followed upon request of the subject, report of a persistent headache, or if the subject appeared extremely uncomfortable although willing to continue.

RESULTS

Constant rate infusions of epinephrine or norepinephrine were characterized by the attainment of steady state plasma concentrations as demonstrated in Figure 1. Although at times fluctuations in the steady state concentrations were observed, particularly for norepinephrine (*cf.* Curves B, C and E), the data in general were indicative of the maintenance of a dynamic balance between the rate at which the particular catecholamine was added to plasma and the rate at which it was simultaneously removed. Steady states were maintained for up to 65 minutes, the longest period tested. For epinephrine infusions, steady

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‡ Deceased July, 1958.

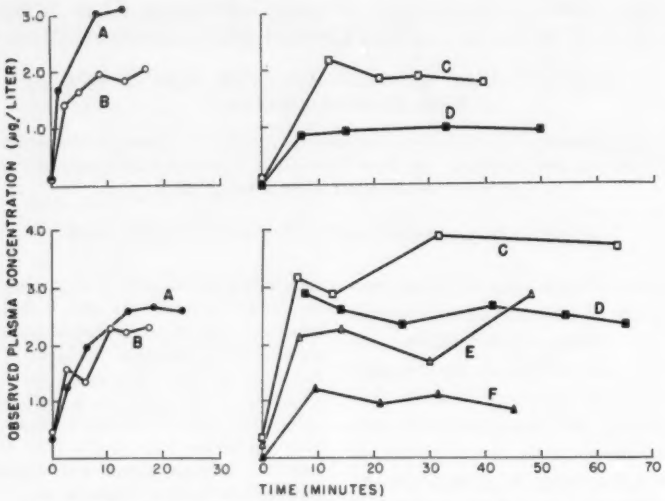

FIG. 1. PERIPHERAL VENOUS PLASMA CONCENTRATION OF CATECHOLAMINE OBSERVED DURING CONSTANT RATE INTRAVENOUS INFUSIONS OF EPINEPHRINE OR NOREPINEPHRINE

Epinephrine infusions (top panel):

A. Normal female subject, S.D., 22 years, 110 lbs., 21.0 μg. per minute.
B. Normal male subject, A.K., 21 years, 175 lbs., 21.0 μg. per minute.
C. Normal male subject, S.T., 24 years, 160 lbs., 20.2 μg per minute.
D. Normal male subject, E.B., 23 years, 165 lbs., 12.4 μg. per minute.

Norepinephrine infusions (bottom panel):

A. Normal male subject, F.S., 23 years, 150 lbs., 21.0 μg. per minute.
B. Normal male subject, M.N., 22 years, 140 lbs., 17.5 μg. per minute.
C. Schizophrenic female subject, J.M., 23 years, 120 lbs., 20.0 μg. per minute.
D. Normal male subject, A.K., 21 years, 175 lbs., 20.8 μg. per minute.
E. Normal male subject, D.H., 22 years, 140 lbs., 21.0 μg. per minute.
F. Schizophrenic male subject, R.Z., 18 years, 130 lbs., 11.6 μg. per minute.

state concentrations were attained within 5 to 10 minutes. For norepinephrine infusions, the time at which the steady state was attained was not always clear due to fluctuations in the observed plasma concentrations; however, roughly 10 minutes or less appeared to have been required.

During the infusion of one catecholamine (either epinephrine or norepinephrine), the changes in plasma concentration of the alternate noninfused catecholamine were small and generally within the limits of accuracy of the analytical procedure. Representative data may be found in Table I where the results of two typical stepwise infusions at successively higher rates are listed. For such infusion studies, blood specimens were drawn at each infusion speed after 10 minutes had elapsed, *i.e.*, at the steady state. Hemodynamic responses to infusions were as previously reported by Goldenberg and co-workers (1). There was considerable variation in intensity of blood pressure and pulse response among the subjects; however, there was no correlation between individual differences in sensitivity and plasma concentrations of the infused pressor amines.

For each individual, the steady state plasma concentration of infused catecholamine was related to the infusion speed in a linear fashion. This is demonstrated in Figure 2 where the results of separate infusions of epinephrine or norepinephrine in the same individual have been graphed. It should be noted that each epinephrine or norepinephrine curve represents a separate infusion of that particular catecholamine. Of particular interest was the finding that for comparable in-

fusion speeds, norepinephrine attained a higher concentration in plasma than did epinephrine.

The steady state data accumulated during all 41 infusions have been plotted in Figure 3. Very little variation among individuals was evident for steady state concentrations of epinephrine; the data lay on a fairly well defined straight line. For norepinephrine infusions, on the other hand, a much wider range of steady state concentrations was observed; however, it was clear that such variations were not due to any inherent difference between the normal and schizophrenic groups. The highest plasma concentrations attained by the infused catecholamines during these studies were in the neighborhood of 6 μg. per L. for norepinephrine infusions and 3 μg. per L. for epinephrine infusions; these values were 10- to 15-fold higher than the highest normal resting values observed, *viz.*, 0.6 μg. per L. for norepinephrine and 0.2 μg. per L. for epinephrine.

TABLE I

*Blood pressure, pulse and peripheral venous plasma concentrations of epinephrine and norepinephrine during intravenous infusions of either catecholamine (Subject L.M.)**

Infusion rate	Time	Blood pressure	Pulse	Plasma concentration	
				Norepin.	Epin.
μg./Kg./min.	*min.*	*mm. Hg*	*per min.*	*μg./L.*	
Norepinephrine infusion					
0.000†	0				
	8	110/80	68		
	11			0.16	0.02
0.100	13				
	23	120/80	54		
	23.5			0.47	0.19
0.203	25				
	35	140/90	48		
	36			2.14	0.23
0.360	37.5				
	47.5	160/90	52		
	48			4.43	0.36
0.472	50				
	58.5	170/90	52		
	61			6.06	0.36
Epinephrine infusion					
0.000†	0				
	9	110/80			
	10			0.21	0.05
0.094	12				
	21	140/70	96		
	22.5			0.12	0.63
0.188	25				
	34.5	140/60	108		
	35.5			0.07	1.09
0.313	37				
	46	140/70	108		
	46.5			0.03	1.90

* Schizophrenic female, 30 years, 140 lbs.
† Saline infusion.

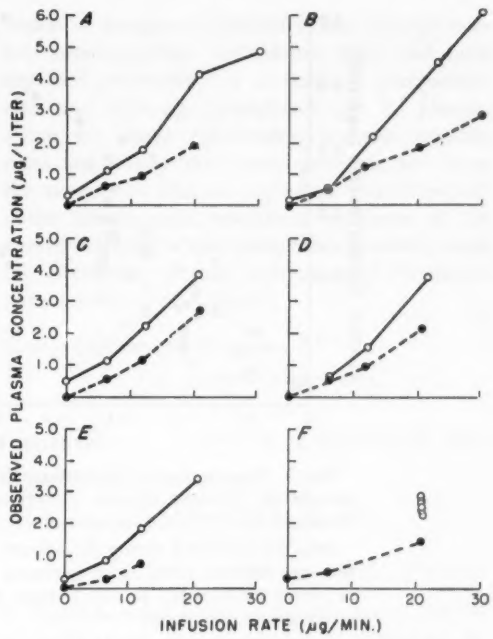

FIG. 2. COMPARISON OF SEPARATE INFUSIONS OF EPINEPHRINE OR NOREPINEPHRINE IN SEVERAL SUBJECTS

Plasma concentrations were measured for each infusion speed after 10 minutes had elapsed, *i.e.*, at the steady state. The concentration of only the infused catecholamine was plotted. Epinephrine is represented by the dashed lines with solid circles; norepinephrine by the solid lines with open circles.

A. Schizophrenic male subject, J.T., 33 years, 155 lbs.
B. Schizophrenic female subject, L. M., 30 years, 140 lbs.
C. Normal male subject, A.F., 21 years, 185 lbs.
D. Normal male subject, R.D., 23 years, 140 lbs.
E. Normal male subject, D.P., 22 years, 150 lbs.
F. Normal male subject, A.K., 21 years, 175 lbs. The norepinephrine data for this subject were obtained during a prolonged constant rate infusion.

In several studies, blood specimens were drawn at various time intervals after cessation of the infusion in order to determine the rate of decay of infused catecholamine from plasma. The data obtained are listed in Table II. The decay from plasma was very rapid with a half-life of roughly one to two minutes (*cf.*, Subjects 2, 3, 4 and 6).

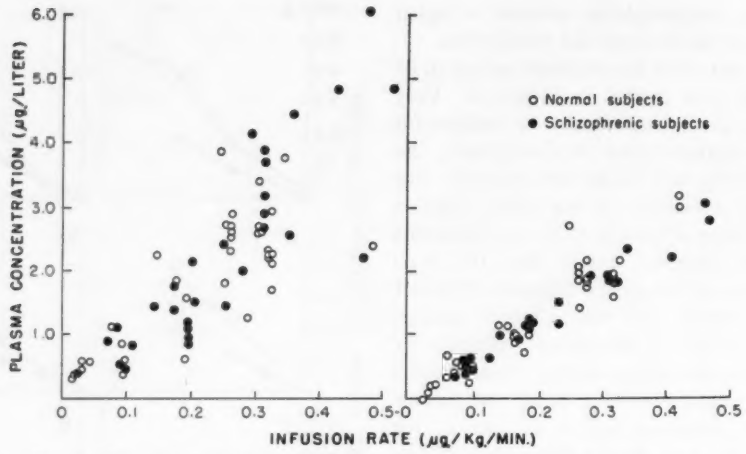

FIG. 3. STEADY STATE PERIPHERAL VENOUS PLASMA CONCENTRATIONS OF CATECHOLAMINE OBSERVED DURING CONSTANT RATE INTRAVENOUS INFUSIONS OF EPINEPHRINE (RIGHT) OR NOREPINEPHRINE (LEFT)

Data were obtained during 41 separate infusions of epinephrine or norepinephrine. For each infusion speed, blood specimens were drawn after 10 minutes had elapsed, *i.e.*, at the steady state. In each instance, the plasma concentration of only the infused catecholamine was plotted.

Norepinephrine plasma levels returned to normal within four to eight minutes (*cf.*, Subjects 4, 5 and 7). In a single instance, *viz.*, Subject 1, there appeared to be a mild elevation in the epinephrine level 10 minutes after cessation of the infusion.

TABLE II

In vivo *rate of decay of infused catecholamines from plasma*

Subject	Infused catecholamine	Sampling time after infusion halted	Catecholamine concentration in plasma
		min.: sec.	*μg./L.*
1	Epinephrine	0:00*	2.79
		10:00–11:00	0.39
2	Epinephrine	0:00*	2.70
		1:00– 1:40	1.62
3	Epinephrine	0:00*	0.71
		1:00– 1:50	0.47
	Norepinephrine	0:00*	3.42
		1:00– 1:50	2.50
4	Norepinephrine	0:00*	2.68
		1:30– 3:00	0.72
		7:00– 8:00	0.28
5	Norepinephrine	0:00*	2.43
		4:00– 5:30	0.49
6	Norepinephrine	0:00*	1.47
		0:30– 1:30	1.25
7	Norepinephrine	0:00*	0.86
		4:00– 5:30	0.25

* Sample drawn at steady state, just previous to cessation of infusion.

DISCUSSION

During a constant rate infusion, the steady state represented a condition in which the rate of addition of catecholamine to plasma was exactly equal to the summation of the rates of the various processes regulating the disappearance of that catecholamine from plasma (*viz.*, diffusion, excretion, metabolic transformation, binding to various tissues and so forth). Although the major source of the elevated plasma catecholamine levels was obviously the infused material, consideration was also given to the possibility of increased endogenous contributions from the body stores such as the adrenal glands and sympathetic nerves. However, the data indicated a lack of significant contributions from these sources. For instance, during norepinephrine infusions the changes in plasma concentration of epinephrine were small (Table I) indicating no major adrenal participation. Similarly, no norepinephrine influx could be detected during epinephrine infusions.

The removal of catecholamine from plasma during infusions was associated with overall first order kinetics as demonstrated by the linearity between steady state concentration and infusion rate (Figure 2). No saturation of the mechanisms

controlling catecholamine removal from plasma was detected for plasma levels as high as 6 μg. norepinephrine per L. or 3 μg. epinephrine per L., *i.e.*, linearity was maintained at these levels. The remarkable degree to which all subjects exhibited similar abilities to remove epinephrine from plasma during infusions was demonstrated by the narrow limits between which the data fell in Figure 3. The wider scattering of the norepinephrine data was no doubt due, at least in part, to the fluctuations in steady state concentrations which were observed at times during constant rate infusions, and also perhaps to individual differences among the subjects. The lack of any difference between data obtained from normal and schizophrenic subjects (Figure 3), and the relevance of this observation to current suggestions of defects in epinephrine metabolism associated with schizophrenia, are discussed in greater detail elsewhere (6).

The relatively higher concentrations attained by norepinephrine than by epinephrine during infusions at equivalent rates (Figure 2) indicated that epinephrine was removed from circulation at a faster rate than norepinephrine. However, it should be noted that the strong hemodynamic effects of infusions of epinephrine or norepinephrine may have affected the steady state concentrations. For instance, epinephrine infusions are known to increase blood flow through liver whereas norepinephrine infusions do not (3). Since the liver has been demonstrated to be a major site for epinephrine and norepinephrine inactivation (7), it follows that increased liver blood flow associated with infusions of epinephrine may have resulted in lower steady state concentrations. Other hemodynamic factors such as the increased cardiac output and decreased total peripheral resistance associated with infusions of epinephrine, and the increased total peripheral resistance associated with infusions of norepinephrine (1) may also have been contributory.

Estimation of resting secretion rates

It may be observed in Figure 3 that average steady state plasma concentrations of roughly 3.5 μg. norepinephrine per L. or 2.5 μg. epinephrine per L. were associated with infusion rates of 0.4 μg. per Kg. per minute. In the absence of infusion of exogenous catecholamine, average normal concentrations (antecubital vein) had been reported previously (5) as 0.30 μg. norepinephrine and 0.06 μg. epinephrine per L. plasma. From the linear relationship between infusion rates and steady state concentrations, and from the assumption that resting values were observed under steady state conditions, estimates of the average rates at which endogenous material reach the circulating plasma under resting conditions were calculated as follows:

$$\text{Norepinephrine, } 0.35 \text{ μg. per L.} \times \frac{0.4 \text{ μg. per Kg. per min.}}{3.5 \text{ μg. per L.}} = 0.04 \text{ μg. per Kg. per min.}$$

$$\text{Epinephrine, } 0.06 \text{ μg. per L.} \times \frac{0.4 \text{ μg. per Kg. per min.}}{2.5 \text{ μg. per L.}} = 0.01 \text{ μg. per Kg. per min.}$$

It should be noted however, that the major portion of endogenous norepinephrine is produced by sympathetic nerves (8, 9) which do not secrete directly into the general circulation; hence, only that portion escaping metabolic destruction or binding between the sites of secretion and subsequent entry into the blood circulatory system is reflected in the plasma concentration. Therefore the average endogenous self-infusion rate calculated for norepinephrine should be distinguished from the average endogenous secretion rate, for which the self-infusion rate represents a lower limit. In comparison, the major portion of endogenous epinephrine enters the circulation directly via the adrenal veins, and hence the endogenous secretion rate for epinephrine may be calculated directly as shown.

TABLE III

Calculated secretion rates of chromaffin tumors (pheochromocytoma)

Patient	Plasma concentration		Calculated secretion rate
	Norepin.	Epin.	
	μg./L.		μg. norepin./Kg./min.
J.T.	1.9	0.17	0.22
K.H.	3.2	0.14	0.37
S.G.	5.6	0.0	0.64
P.L.	12.2	0.4	1.4
J.B.	30.5	0.2	3.5

The infusion experiments simulated very closely the situation in patients with norepinephrine and/or epinephrine producing tumors, *i.e.*, pheochromocytomas. Secretion rates of such tumors could therefore be calculated in the same manner as outlined for resting secretion rates. Several examples of the plasma concentrations observed by us in patients with pheochromocytomas and of the secretion rates to which they corresponded are listed in Table III.

Calculation of turnover times

The "turnover time" of a hormone has been defined (10) as the time required for complete replacement of circulating hormone by a fresh endogenous supply. At the steady state, the turnover time is given by the equation $T = cv/r$, where c is the average concentration of hormone in plasma, v is the total volume of plasma, and r is the average rate at which hormone is added to blood. For plasma epinephrine and norepinephrine (antecubital vein), a turnover time of 30 seconds was calculated from average steady state concentrations (v) of 3 μg. per L. associated with infusion rates (r) of 20 μg. per minute (Figure 2), and from an average plasma volume (v) of 3.3 L. (based on an average blood volume of 6 L. and an average plasma composition of 55 per cent). Actually the value for epinephrine should be some 20 per cent lower while that for norepinephrine should be some 20 per cent higher than the calculated value on the basis of the relatively lower or higher steady state concentrations observed for each substance, respectively. In comparison the following turnover values in whole blood have been calculated by Pearlman (10): 3.3 minutes for progesterone, 6 minutes for total estrogens, and 17 minutes for 17-hydroxycorticoids. It should be noted that the turnover time calculated for the infused catecholamines must be the same as that for endogenous secreted pressor amine in view of the linear relationship between infusion rate and steady state concentration (Figures 2 and 3).

SUMMARY

1. Peripheral venous plasma concentrations of epinephrine and norepinephrine were measured during intravenous infusions of these substances. During infusions, concentrations as high as 3 μg. epinephrine or 6 μg. norepinephrine per L. plasma were attained.

2. Constant rate infusions were characterized by the attainment of steady state plasma concentrations within 5 to 10 minutes. Steady state concentrations were directly proportional to infusion speeds. Higher concentrations were attained by norepinephrine than by epinephrine for infusions at comparable speeds.

3. There was very little variation among individuals (both normal and schizophrenic) with regard to rates at which epinephrine was removed from plasma during infusions. Wider variations were noted for norepinephrine.

4. The half-life in plasma of the infused catecholamines was about one to two minutes.

5. Normal endogenous self-infusion rates of 0.04 μg. norepinephrine per Kg. per minute, and 0.01 μg. epinephrine per Kg. per minute were calculated from the data. Similar calculations were employed to determine the secretion rates in patients with pheochromocytoma.

6. Turnover times of 30 seconds were calculated for plasma epinephrine and norepinephrine.

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SERUM INHIBITION OF STREPTOCOCCAL DIPHOSPHOPYRIDINE NUCLEOTIDASE IN UNCOMPLICATED STREPTOCOCCAL PHARYNGITIS AND IN RHEUMATIC FEVER *

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One of the recently described extracellular products of Group A streptococci is diphosphopyridine nucleotidase (DPNase) (1). This enzyme has received attention as a substance of possible importance in the etiology of either rheumatic fever or glomerulonephritis because *a*) it was isolated from cardiotoxic fractions of supernates of broth cultures of Group A streptococci (2, 3) and *b*) almost all nephritogenic strains of Group A streptococci studied produced it *in vitro* (4).

Kellner, Freeman and Carlson (5) showed that the inhibition of DPNase by human sera of different age groups follows the pattern of other streptococcal antibodies and that DPNase neutralizing titers increase in response to streptococcal infection.

Studies in progress in this laboratory on rheumatic fever and the human immune response to streptococcal infection afforded the opportunity to compare the behavior of the antibody response to DPNase with antibody responses to some other known antigenic extracellular enzymes of the streptococcus. This report presents further evidence that antistreptococcal DPNase activity (ASDA) of human sera behaves as a specific antibody and that it reflects streptococcal disease in rheumatic and nonrheumatic individuals in a manner very similar to antistreptolysin O, antihyaluronidase and antistreptokinase titers.

MATERIALS AND METHODS

The method employed for the assay of DPNase and antiDPNase was based upon that described by Carlson, Kellner, Bernheimer and Freeman (1), with some modifications. Streptococcal DPNase activity was measured by determining the enzymatic degradation of a standard amount of DPN. ASDA was determined by measuring the inhibition of DPNase activity by serial dilutions of human serum.

Preparation of streptococcal DPNase. Partially purified streptococcal DPNase was prepared in the following manner. The C203S strain of Group A streptococci was grown in Todd-Hewitt broth for 18 hours, penicillin added (approximately 100 units per ml.), and the supernate separated from the streptococcal cells in a Sharples centrifuge at 4° C. The enzyme in the supernate was precipitated with half saturated ammonium sulfate and dialyzed in cellophane bags against running tap water for 20 hours. The solution was concentrated by evaporation in cellophane bags to one-tenth of its original volume and centrifuged to remove precipitate. Aliquots of the supernate were frozen at −70° C. in a dry ice-alcohol bath and stored at −65° C. Prior to use in the assay of ASDA, aliquots of enzyme were thawed and diluted in a 0.1 M phosphate buffer, pH 7.4, containing 0.1 per cent bovine serum. Enzyme activity did not diminish significantly when the buffered solution was kept at 4° C. for several days.

Assay of DPNase activity. DPNase activity was measured as follows. To each of four tubes [1] was added 0.2 ml. of twofold serial dilutions (1:4 to 1:32) of crude enzyme and 0.8 ml. of a 1 mg. per ml. solution of DPN.[2] The mixture was incubated in a water bath of 37° C. for 7.5 minutes. The reaction was stopped by plunging the tubes into an ice bath and promptly adding 6.0 ml. of 1 M sodium cyanide solution freshly prepared. Control tubes in which buffer was substituted for enzyme and for substrate, respectively, were included with each assay. The optical density of the DPN-cyanide complex was read at 340 mμ against a cyanide blank in a photocolorimeter. Originally the Beckman DU spectrophotometer was used. However, the Bausch and Lomb "Spectronic 20" colorimeter was found capable of measuring optical density at 340 mμ with sufficient accuracy for the purposes of this assay and was also employed in this study. One unit of DPNase activity is defined as the amount necessary to destroy 0.01 μMole of DPN at

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[1] Kimball screw cap culture tubes 13 × 75 mm. were found convenient.

[2] Nutritional Chemical Company supplied DPN powder of 95 per cent purity.

37° C. in 7.5 minutes (6). A 1:16 dilution of the crude streptococcal enzyme employed destroyed approximately 0.5 μMole of DPN under these conditions.

Titration of antistreptococcal DPNase (ASDA) in serum. Serum to be tested for ASDA content was diluted serially twofold in phosphate buffer. Dilutions of 1:4 to 1:256 were sufficient to measure the range of antibody titers usually encountered in patients with streptococcal disease. To each tube was added 0.2 ml. of serum dilution and 0.2 ml. of DPNase solution (approximately 250 units). Control tubes containing enzyme without serum antibody, and substrate without enzyme, respectively, were included. All tubes were incubated at 37° C. for 30 minutes to allow for combination of antigen and serum antibody and then were placed in an ice bath. To test residual DPNase activity, 0.6 ml. of DPN solution (1.33 mg. per ml.) was added to each tube and the same procedure followed as described above for the assay of DPNase.

The curve representing decrease in optical density due to enzymatic destruction of DPN is linear only to 60 per cent destruction. Therefore, inhibition of the decrease in optical density along the linear portion of the curve was the range used in the calculation of the ASDA titer. By arbitrary definition, one unit of antibody inhibits 100 units of DPNase (5). The ASDA titer was calculated from the serum dilution resulting most nearly in a 50 per cent inhibition of DPNase as follows: units of DPNase inhibited × reciprocal of serum dilution × 10^{-2} units of antibody are expressed as the reciprocal of the serum dilution titer in the assay of most streptococcal antibodies. The same convention has been observed for expressing units of ASDA.

Other methods employed. Antistreptolysin O (ASO) determinations were made by the method of Rantz and Randall (7); antistreptokinase (ASK) by the method of Christensen (8) and antihyaluronidase (AH), by the method of Harris and Harris (9). Throat cultures were made by inoculating throat swabs directly on 5 per cent sheep blood agar plates. Beta hemolytic streptococcal colonies isolated were grouped and typed by conventional serological methods (10). All sera were frozen and stored at −20° C. or were placed in tightly capped vials and stored at 4° C. Serum was clarified by centrifugation before determination of the ASDA titer.

RESULTS

Specific antibody properties of ASDA

In the preliminary stages of this study and before additional data of others were available, it was considered of importance to determine whether or not the streptococcal DPNase inhibitor in human serum had the properties of a specific antibody. In distinction to most nonspecific serum inhibitors, antibodies that neutralize streptococcal enzymes are relatively thermostabile γ-globulins and are usually specific for the species from which the enzyme is derived.

TABLE I

Replicate determinations of streptococcal antibodies in a pool of human serum

Streptococcal antibody*	No. of determinations	Mean titer	Standard deviation
		units/ml.	*units/ml.*
ASDA	98	64	4.7
ASO	58	127	6.4
AH	33	136	19.3
ASK	27	80	4.0

* ASDA = antistreptococcal diphosphopyridine nucleotidase; ASO = antistreptolysin O; AH = antihyaluronidase; ASK = antistreptokinase.

Accordingly, some pools of human sera were studied for these properties. ASDA was unaffected by heating serum at 56° C. for one hour. More than 95 per cent of ASDA activity recovered was localized in the fraction of serum precipitated by 0.33 ammonium sulfate saturation. Euglobulin fractions prepared in our laboratory from pools of human sera, and several commercial lots [3] of human γ-globulin that were known to be high in ASO, ASK and AH activity, were also found to be relatively high in ASDA.

The species specificity of human serum inhibition of streptococcal DPNase was also confirmed. DPNase was prepared from beef spleen and was tested in parallel determinations with streptococcal DPNase against sera of known high ASDA titer. Beef spleen DPNase was not inhibited at all by sera that strongly inhibited streptococcal DPNase.

The reproducibility of the ASDA test

The ASDA titration was compared with three other well known streptococcal antibody determinations (Table I). Replicate determinations were made on the same pool of human serum: 98 times for ASDA; 58 times for ASO; 33 times for AH; and 27 times for ASK. The geometric mean titers and standard deviations were as follows: ASDA, 64 units per ml., ± 4.7; ASO, 127 units per ml., ± 6.4; ASK, 80 units per ml., ± 4.0; and AH, 136 units per ml., ± 19.3.

The ASDA titers were calculated as actual units of enzyme inhibited per milliliter of serum rather than, as in the case of the other antibodies, as the

[3] Cohn Fraction II, supplied by Hyland Laboratories, Los Angeles, Calif.

TABLE II

Relative behavior of antistreptococcal diphosphopyridine nucleotidase (ASDA) and known specific antibodies in streptococcal disease

Group	No. of patients	Geometric mean titer			
		ASDA	ASO	AH	ASK
		units/ml.	*units/ml.*	*units/ml.*	*units/ml.*
Acute pharyngitis	87	84	227	101*	36*
Conval. pharyngitis	87	226	468	180*	160*
Acute rheum. fever	31	358	497	578	173
Inactive rheum. fever†	18	33	41	60	30

* The AH and ASK titers of only 25 of 87 patients with pharyngitis were studied.
† Adolescents or adults with inactive rheumatic fever for five or more years and known to be free of streptococcal infection for at least one year before this assay.

serum dilution closest to an arbitrary end point of the test. An increase in the ASDA titer of more than three times the standard deviation, or greater than 22 per cent was, therefore, considered significant when serum samples obtained from serial bleedings were tested simultaneously.

Behavior of ASDA following acute streptococcal pharyngitis

Eighty-seven school children who had symptoms and signs of uncomplicated acute pharyngitis, associated with throat cultures positive for Group A streptococci, were studied. Seventeen were treated with one injection of 600,000 units of benzathine penicillin intramuscularly and the remainder received symptomatic therapy only.[4] Serum was collected at the initial visit during the acute stage of pharyngitis and at 21, 35 and 60 days thereafter. ASDA and ASO determinations were made on paired samples of acute and convalescent phase sera in each patient.

The streptococcal antibody response was analyzed in three ways: *a*) the geometric mean of the titers of all patients' acute phase sera was compared with that of all patients' convalescent phase sera; *b*) the per cent of patients whose paired sera showed a significant increase in antibody titer during convalescence was determined; and *c*) the per cent of patients whose *acute* phase sera showed high titers (see below), without subsequent increase during convalescence, was determined.

The geometric mean of ASDA titers in acute and convalescent phase sera was 84 and 226 units per ml., respectively. The geometric mean of ASO titers in acute and convalescent phase sera was 227 and 468 units per ml., respectively. The sera of 25 of the 87 patients with pharyngitis were titrated for AH and ASK. There was similarly an increase in the mean titer of these antibodies during convalescence (Table II).

Significant increase in the titer of ASDA during

[4] These patients were part of a controlled study of the treatment of streptococcal pharyngitis in school children now in progress at Children's Memorial Hospital in Chicago (11).

TABLE III

*Comparison of antibody titers in paired acute and convalescent sera of 87 patients with streptococcal pharyngitis**

Streptococcal antibody	Magnitude of rise in titer		Per cent of patients with:		
				No rise in titer	
	Mean	Range	Rise in titer	Acute phase increased†	Acute phase not increased
	units/ml.	*units/ml.*			
ASDA	197	20–1,970	63	9	28
ASO	454	66–2,400	62	8	30
ASDA or ASO			75	17	8

* Seventeen patients were treated with penicillin. The remainder had mild, uncomplicated infections and received symptomatic treatment only (see text).
† The acute phase sera of these patients had increased titers and the titer of their convalescent sera did not fall. Titers were considered "increased" if ASDA was greater than 200 units per ml. or ASO was greater than 500 units per ml.

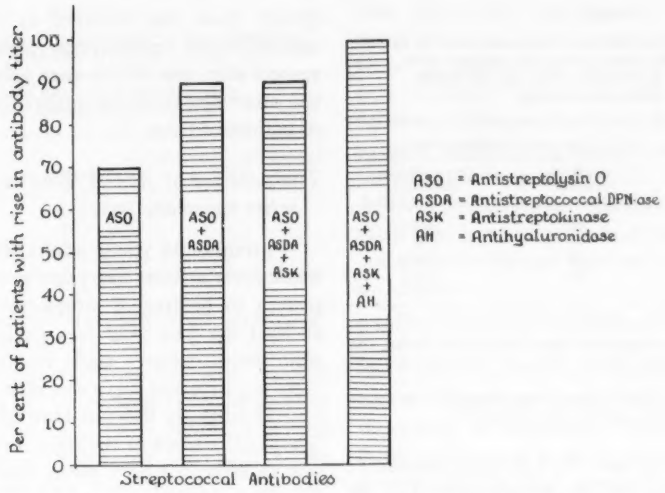

FIG. 1. RISE IN ANTIBODIES FOLLOWING UNCOMPLICATED GROUP A STREPTOCOCCAL PHARYNGITIS IN SCHOOL CHILDREN

convalescence was observed in the sera of 63 per cent of these children. The ASO titer increased in 62 per cent of the same patients. A rise in the titer of *either* ASDA or ASO occurred in 75 per cent of patients (Table III).

Nine per cent of patients exhibited acute phase ASDA titers greater than 200 units per ml. which did not increase or decrease during convalescence. On the basis of distribution curves made for streptococcal antibody titers in this group of patients, levels of 200 units of ASDA were considered to be approximately comparable to 500 units of ASO. Eight per cent of patients had initial ASO titers that were greater than 500 units per ml. and did not change during convalescence.

It is often difficult to detect antibody responses to infection in patients when serum levels are already considerably increased during the acute phase of the disease. It was therefore necessary to treat the data of such patients as a separate group from those considered to have "no antibody response" and to reserve the latter category for patients whose titers were not at high levels during the acute phase and did not increase during convalescence.

By these criteria 28 per cent of patients with streptococcal pharyngitis had no ASDA response and 30 per cent had no ASO response. Only 8 per cent had no response of *either* titer (Table III).

The relative sensitivity of ASDA to three other antibody tests in acute pharyngitis

Of the 87 school children with acute streptococcal pharyngitis, 32 patients were selected for multiple antibody determinations. The patients selected had uncomplicated acute pharyngitis which subsided promptly without antibiotic therapy. All had throat cultures positive for Group A streptococci. A significant increase in ASO titer occurred in 69 per cent of the group. When determinations of ASDA were made in addition, 90 per cent of patients showed an increase in the titer of *either* antibody. The addition of antistreptokinase measurements in the same sera did not increase the number of significant antibody responses. When the antihyaluronidase determination was also made, however, 100 per cent of the cases showed a significant rise in the titer of at least one of the four antibodies (Figure 1). Of the above group of 32 patients, there were 25 in whom determinations were made of all four streptococcal antibodies in all acute and convalescent sera. Antibody titers increased during convalescence or were elevated initially during the

TABLE IV

Comparison of four streptococcal antibody titers in paired acute and convalescent sera of 25 patients with streptococcal pharyngitis who did not receive penicillin therapy

Streptococcal antibody	Per cent of patients with: Antibody response*	No antibody response†
ASDA	80	20
ASO	92	8
AH	68	32
ASK	40	60

* These patients' sera showed a significant increase in titer during convalescence or showed high titers during the acute phase of illness which did not fall during convalescence.

† These patients' acute phase sera showed low titers which failed to increase during convalescence.

acute phase of illness with the following frequency: ASO, 92 per cent; ASDA, 80 per cent; AH, 68 per cent; and ASK, 40 per cent (Table IV).

Behavior of ASDA in acute rheumatic fever

The sera of 31 young men with acute rheumatic fever [5] had mean ASDA and ASO titers of 358 and 497 units per ml., respectively (Table II). A more complete antibody study of this group of patients with acute rheumatic fever is shown in Table V. Titers were found to be increased above usual levels for this age group with the following frequency: ASDA, 87 per cent (130 units per ml. or greater); ASO, 87 per cent (250 units per ml. or greater); AH, 76 per cent (256 units per ml. or greater); and ASK, 63 per cent (160 units per ml. or greater). These so-called "upper normal limits" were approximately twice the titers encountered in pools of sera from healthy individuals in this age group.

A combination of ASO and ASDA titers showed increased levels of *either* antibody in 94 per cent of the rheumatic fever patients' sera. Combination of the ASO with either the ASK or the AH test also detected increased levels of antibodies in 94 per cent of patients. When all four antibodies were determined, all patients showed an increase of at least one of the four titers.

It is noteworthy that the mean ASDA titer in the acute rheumatic fever patients was somewhat greater than that observed in the patients with uncomplicated streptococcal pharyngitis. In this respect also, the ASDA titer behaved similarly to the other streptococcal antibodies in both groups of patients (Table II).

[5] These sera were kindly supplied by the staff of Naval Medical Research Unit No. 4, Great Lakes Naval Training Center, Great Lakes, Ill.

The behavior of ASDA titers in patients with inactive rheumatic fever

A group of 18 young adults who had had rheumatic fever at least five years previously and were proven to be free of streptococcal infections for at least the past year was studied. During this time throat cultures made routinely at each visit were negative and there was no rise in ASO, ASK or AH antibody titers at bimonthly examinations. The ASDA titers of the same sera were uniformly low as indicated by a mean titer of 33 units per ml. (Table II).

In vitro compared with in vivo DPNase production of strains of Group A streptococci

The *in vitro* production of DPNase by Group A streptococci isolated from patients with pharyngitis was related to the ASDA antibody response in the sera of the same patients. Sixteen strains were recovered and cultured in Todd-Hewitt broth. The culture supernates were analyzed for DPNase activity by the method of Bernheimer, Lazarides and Wilson (4). Six strains did not produce DPNase *in vitro*. These included Types 1, 3, 12 and 19. Ten strains of streptococci produced DPNase in broth. These included Types 6, 12 and two "untypable" strains. There was a significant ASDA response in four of the six patients from whom "non-DPNase producing organisms" were recovered and in 7 of 10 pa-

TABLE V

Antibody titers in 31 patients with acute rheumatic fever

Streptococcal antibody	Per cent increased*	Geometric mean	Range
		units/ml.	*units/ml.*
ASDA	87	358	25–2,000
ASO	87	497	50–1,250
AH	76	578	16–8,000
ASK	63	173	20–1,280

* Titers considered higher than the usual range encountered in this age group in the absence of known recent infections were as follows: ASDA, 130 units per ml. or more; ASO, 250 units per ml. or more; AH, 256 units per ml. or more; ASK, 160 units per ml. or more.

TABLE VI

In vitro production of diphosphopyridine nucleotidase (DPNase) by 16 strains of Group A streptococci isolated from 16 patients with pharyngitis whose ASDA titers were determined in acute and convalescent phase sera

In vitro DPNase production	No. of strains	No. of patients with increase in ASDA
Positive	10	7
Negative	6	4
Total	16	11

tients from whom "DPNase producing organisms" were cultured (Table VI).

Of particular interest were the four patients whose strains failed to produce DPNase *in vitro* but whose sera showed a rise in ASDA following infection. Of these strains, one was Type 3, one was Type 12 and two were Type 1. No significant correlation was observed, therefore, between *in vitro* and *in vivo* production of DPNase.

DISCUSSION

The data presented confirm previous observations that ASDA activity of human serum has characteristic properties of specific streptococcal antibody. In common with most antibodies ASDA is stable upon heating at 56° C., is associated with the γ-globulin fraction of serum and is species specific in its inhibition. Its failure to neutralize DPNase derived from beef spleen is in accord with the observations on species specificity by Kellner, Freeman and Carlson (5) who showed that ASDA could not neutralize DPNase derived from *Neurospora crassa*. The latter authors also showed that multiple injections of streptococcal DPNase in rabbits resulted in the appearance of progressively high titers of ASDA in their sera.

The data presented in this study demonstrate closely parallel behavior of ASDA titers and of ASO, AH and ASK titers following proven streptococcal infections. The behavior of the latter three antibodies was similar to that observed in other studies (12–15). The ASDA titer seemed to be approximately as sensitive as the ASO and somewhat more sensitive than AH and ASK titers in detecting antecedent streptococcal disease.

No peculiarity or atypical behavior of ASDA was noted in patients with acute rheumatic fever. Like the other streptococcal antibody studies, mean titers of ASDA were somewhat higher in acute rheumatic fever patients' sera than in the sera of patients with uncomplicated streptococcal infection. This tendency of the rheumatic host to show greater streptococcal antibody levels has been reported repeatedly (13). The behavior of ASDA confirms the hyperimmune response of rheumatic fever patients to streptococcal products but sheds no further light on the pathogenesis of the disease.

The determination of the relative frequency of "increased" levels of various streptococcal antibody titers is only an estimate. There is, of course, no absolute level of antibody beyond which titers may be considered abnormally high. The range of titers of a given population varies with geographical location, age and season. The critical levels above which titers were regarded as "increased" in acute phase sera in this study were arbitrarily set at titers that are not usually exceeded in comparable populations free of recent streptococcal disease (12, 16). These levels were twice the titers of pooled sera obtained from such populations. It is acknowledged that the order of frequency of "increased" levels of the various antibodies will vary somewhat if different critical limits are assigned to one or the other. The purpose of this study, however, was more to determine the relative pattern of behavior of ASDA to other antibodies than to determine the "superiority" of one to the other as a test for antecedent streptococcal disease. The similarity of the behavior of ASDA to the other antibodies is more to be stressed than minor differences.

When antibody titers are already at high levels prior to streptococcal infection, it is frequently difficult to detect a further increase following reinfection. This is especially true because the methods employed for measuring antibodies can detect reliably only relatively large increments in titer (0.2 log titer). This difficulty is often encountered when ASO is measured in a population heavily cross infected with Group A streptococci (16). Thus, a patient whose initial ASO titer is 1,000 units per ml. may sustain a new streptococcal infection which increases his titer to 1,250 units per ml. This represents an increment of approximately 0.1 log titer whereas the method commonly employed cannot detect an increment of less

than 0.2 log titer with reliability. The same arithmetic increment of 250 units would be readily measured if the initial ASO titer were low.

In this study, therefore, patients were classified as "no antibody response" when their acute phase titer was not at high levels and when they failed to demonstrate an increase in titer during convalescence (Tables III and IV).

Poor correlation was observed between *in vitro* production of DPNase and the ASDA response *in vivo*. A similar situation exists in the case of streptococcal hyaluronidase (17). Lazarides and Bernheimer found that extracellular production of DPNase was limited to certain strains of streptococci among a large variety of bacterial species studied. Fifty-five per cent of the streptococcal strains tested produced DPNase *in vitro* and these were distributed mostly among Serotypes 3, 4, 6 and 12. Types 1, 5, 14, 19 and 24 usually did not produce the enzyme in broth cultures (18).

In the present study, the type distribution of the four strains that failed to produce DPNase *in vitro* but stimulated ASDA responses *in vivo* was of interest. Two were of types usually associated with good "DPNase producers" (Types 3 and 12) and two were strains of Type 1, usually associated with poor "DPNase producers." Apparently strain variation, with regard to DPNase *in vitro* production, occurs within the same serotype and, in addition, DPNase formation in broth cannot be used to predict the elaboration of this enzyme in the human host.

The ASDA antibody determination appears to be an accurate and relatively practical test for the study of human streptococcal disease. In many ways the system and the technical procedure are less complicated than either the antistreptokinase or antihyaluronidase determinations and some of the reagents are better defined and more readily available. It offers no particular advantage over the ASO titration but should be a useful adjunct to it in increasing the accuracy of diagnosis of antecedent streptococcal infection.

SUMMARY

Serum antistreptococcal diphosphopyridine nucleotidase activity (ASDA) was studied in patients with rheumatic fever and in those with uncomplicated streptococcal pharyngitis, and was compared with the behavior of antibodies to other known streptococcal extracellular enzymes.

Serum ASDA showed characteristic properties of a serum antibody: it was relatively heat stable, associated with γ-globulins, and species specific in its inhibition.

ASDA behaved in closely parallel fashion to other streptococcal antibodies tested in human sera. Increased ASDA was found approximately as frequently as increased antistreptolysin O and somewhat more frequently than increased antihyaluronidase or antistreptokinase titers.

Like the other streptococcal antibodies studied, mean ASDA titers were somewhat higher in acute rheumatic fever patients' sera than in the sera of patients with uncomplicated streptococcal infection. After prolonged freedom from streptococcal infection, ASDA titers were uniformly low in rheumatic subjects. There was, therefore, no evidence of atypical behavior of ASDA in rheumatic fever compared with other streptococcal antibodies.

Increases in ASDA titers in the sera of patients convalescent from streptococcal pharyngitis were not always associated with *in vitro* production of diphosphopyridine nucleotidase by the infecting strain, and *vice versa*.

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DISTRIBUTION AND EXCRETION OF FOUR TETRACYCLINE ANALOGUES IN NORMAL YOUNG MEN *

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Three tetracycline analogues, tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC) have been used extensively in man and are well established as effective antimicrobial agents. Recently a fourth analogue, demethylchlortetracycline (DMCT) was described (1) and is being introduced for clinical use.[1] The structural formulas of these four tetracyclines are shown in Figure 1. Some of the important properties of the new analogue are: its resistance to heat, stability in acid and alkaline solution (1), good absorption with well-sustained blood levels (2–4) and antibacterial activity comparable to the best of the previously available analogues (1–5) and, in many instances, superior to them (5). The half-life of DMCT in serum following an oral dose was found to be 44 per cent longer than that of TC; this was shown to be related to the slower rate of renal excretion of the former (2). Thus, DMCT was shown to produce much higher and better sustained levels of antibacterial activity in the serum than either TC (2, 3), CTC (3, 4) or OTC (4).

The antibacterial spectra of the first three tetracyclines have been found to be identical, although some differences in their relative activity against certain organisms have been noted (5–12); the same is true for DMCT (5). An appreciation of the differences among the tetracyclines with respect to activity, physical properties, stability, solubility, plasma protein binding and absorption, distribution and excretion *in vivo* and toxicity should help to determine which, if any, may be superior for any particular clinical situation. The present report presents a comparison of the distribution and excretion of the four analogues as observed in the same four healthy young men following single intravenous injection of each of them. The binding of these analogues to plasma protein was also determined *in vitro*.

MATERIALS AND METHODS

Tetracycline analogues. Sterile ampules labeled as containing 500 mg. of the hydrochloride of TC and CTC and 250 mg. of DMCT–HCl were provided by Lederle Laboratories Division of American Cyanamid Co., and similar ampules of OTC–HCl, labeled as containing 500 mg. base equivalent, were furnished by Charles Pfizer and Co. Each of these preparations was buffered with ascorbic acid except the CTC which was buffered with sodium glycinate. Crystalline preparations of each of the analogues were also made available for standard solutions and for studies of plasma protein binding. For intravenous injection, each dose was dissolved in 150 ml. of 5 per cent aqueous solution of dextrose and given over a period of 15 to 20 minutes. Two ampules of each of the lots used were assayed for total drug content and the results are shown in Table I. In the text and tables that follow, the doses given refer to the labeled potency, but all calculations and comparisons of distribution and excretion are based on the assayed values.

Subjects and design of study. Four healthy young men, ranging in age from 28 to 32 years and in weight from 68 to 71 Kg., were given each of the drugs intravenously in the following sequence: TC, 500 mg.; CTC, 500 mg.; OTC, 500 mg.; DMCT, 250 mg. and DMCT, 500 mg. The individual doses were separated by intervals of one to two weeks in order to insure complete elimination before the next dose was given. Blood specimens were obtained before and at 30 minutes after completion of the injections, at the midpoint of three sequential 30 minute urine collections begun two hours after completion of the injection, and at 4, 6, 8, 12, 24 and 30 hours. The serums were separated as soon as possible and stored at −20° C. until the time of the assays.

All urine excreted during the 96 hours after the beginning of the injection was collected. During each col-

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[1] Under the trademark Declomycin®.

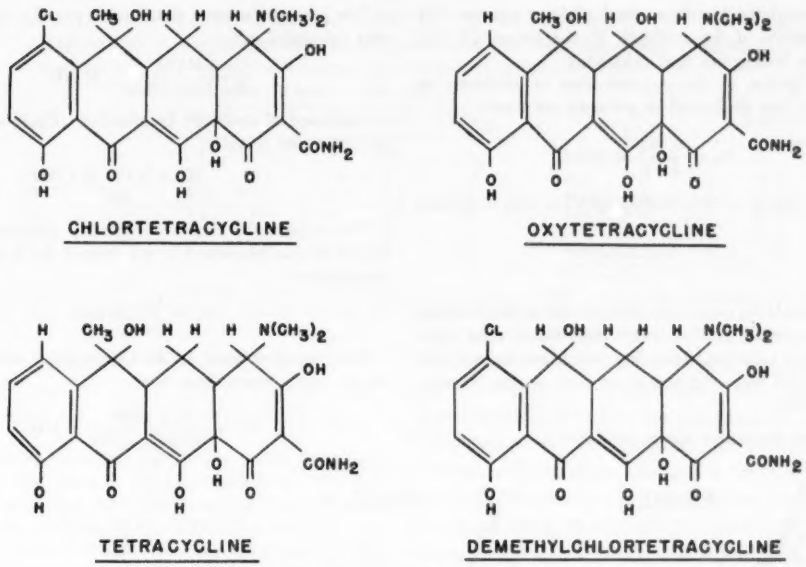

FIG. 1. STRUCTURAL FORMULAS OF FOUR TETRACYCLINE ANALOGUES

lection period, the specimens were refrigerated at 4° C.; at the end of each period these were pooled and aliquots were removed and stored at −20° C. until the time of the assays. Except for the initial two hour period and the three 30 minute periods of the clearance studies, all urines collected from each subject during each 24 hour period were pooled into a single specimen.

Assays of tetracycline analogues. Assays for antibiotic activity in serum were performed by the cup-plate method using *Bacillus cereus* var. *mycoides* ATCC 9634 as test organism (13); the antibiotic content of urine was assayed by the turbidimetric method using *Staphylococcus aureus* ATCC 6538-P (13). Results of each assay were recorded as the mean of two separate determinations done on each specimen, and standard solutions of all four analogues were included with each assay. All assays were carried out in the Research Laboratories of American Cyanamid Co. to which the specimens were delivered in the frozen state under code designations.

Renal clearance. The subjects were given adequate fluids during the first four hours of each study in order to maintain a urine flow of 4 to 12 ml. per minute. Beginning two hours after the end of the injection, three sequential 30 minute urine collections were made without catheterization and blood samples were drawn midway in each period. One of these blood specimens and an aliquot of urine were analyzed for creatinine content [2] by a modification of the method of Bonsnes and Taussky (14).

Calculations. Renal clearance (C) of antibiotic (C_{ar}) or of creatinine (C_{creat}) was calculated from the standard formula (15). Standard deviation (S. D.) was calculated from the values for each of the 30 minute periods (16).

When the concentrations of antibiotic in the serum following intravenous injection were plotted on semilogarithmic paper, two rates of decline were noted: an initial rapid fall followed by a slower one. The second slope

TABLE I

*Results of assays of tetracycline analogues used for intravenous injection in this study**

Antibiotic (HCl)	Labeled potency	F.D.A. assay†	Per cent of labeled potency†
	mg./vial	*mg./vial*	
TC	500	570	114
OTC	500	448 (484)	90 (97)
CTC	500	678	135
DMCT	250	286	114

* TC = tetracycline; OTC = oxytetracycline; CTC = chlortetracycline; DMCT = demethylchlortetracycline.

† Assays were performed by the *Staphylococcus aureus* turbidimetric method in the Antibiotics Division, Food and Drug Administration, through the courtesy of Dr. Henry Welch and Dr. William W. Wright. Both labeled and assayed values were calculated as hydrochloride except those for OTC which are given in terms of the base; values for the corresponding hydrochloride of OTC are given in parentheses.

The values are given in terms of the same antibiotic. Comparisons of the activity of the standard solutions of the four antibiotics indicated that, on a weight basis, DMCT was 90 per cent as active as CTC, 1.9 times as active as OTC and three times as active as TC against the assay organism, *Staphylococcus aureus* ATCC 6539P.

[2] Carried out by Miss Ellen J. Doyle.

(k) was calculated by the method of least squares (2) and the half-life of the antibiotic in the serum ($T_s/2$), expressed in hours, was then calculated.

Rate of decline in the concentration of antibiotic in serum (K_s) was expressed as per cent per hour:

$$K_s = \frac{0.693}{T_s/2} \times 100.$$

Relative volume of distribution (RVD) was expressed in liters:

$$RVD = \frac{\text{mg. injected}^3}{C_0},$$

where C_0 equals the expected concentration, in micrograms of antibiotic per milliliter of serum immediately after completion of the injection, assuming instantaneous mixture, and determined from the second or slow decline of antibiotic in serum.

[3] Based on the assays shown in Table I.

The per cent volume distribution per Kg. body weight was calculated as:

$$\frac{RVD}{\text{Kg. body weight}} \times 100.$$

Clearance of antibiotic from serum (C_{as}) was given in milliliters per minute:

$$C_{as} = \frac{K_s \times RVD \times 1{,}000}{60}.$$

The expected (calculated) urinary recovery (R_e) of antibiotic was expressed in per cent of the actual amount administered:

$$R_e = \frac{C_{ar}}{C_{as}} \times 100.$$

The rate of removal by the kidney (K_r) was expressed as per cent extracted per hour:

$$K_r = \frac{C_{ar} \times 60}{RVD \times 1{,}000} \times 100.$$

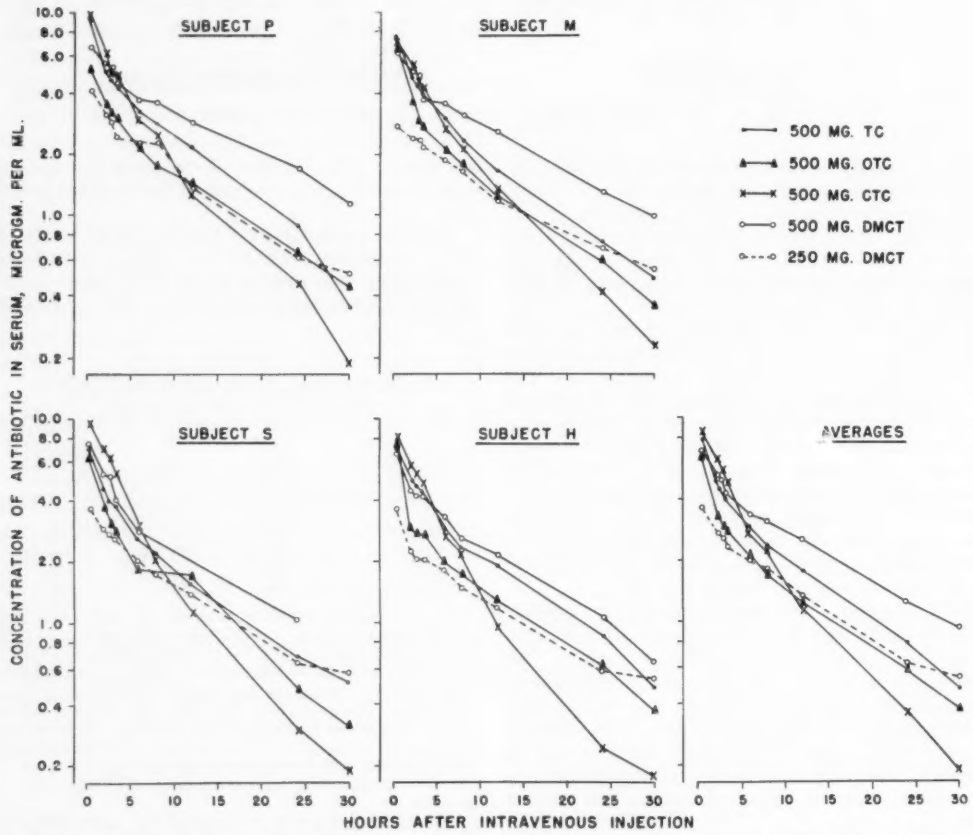

FIG. 2. CONCENTRATIONS OF FOUR TETRACYCLINE ANTIBIOTICS IN SERUM OF FOUR NORMAL MEN AFTER SINGLE INTRAVENOUS INJECTIONS

The levels are plotted on a logarithmic scale and are given in terms of the antibiotic that was administered.

TABLE II

*Disappearance of four tetracycline analogues from serum after single intravenous injections**

Subject	Weight	Height	Rate of decay in serum (K_s)					Half-life in serum ($T_s/2$)				
			TC	OTC	CTC	DMCT	$DMCT_{250}$	TC	OTC	CTC	DMCT	$DMCT_{250}$
	Kg.	*cm.*	*%/hr.*	*%/hr.*	*%/hr.*	*%/hr.*	*%/hr.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
H	68.0	177	7.9	7.3	13.5	6.2	5.4	8.8	9.5	5.1	11.2	12.9
M	70.5	170	8.2	7.7	11.0	5.2	5.6	8.4	9.1	6.3	13.3	12.4
P	61.5	177	9.1	7.2	11.3	4.9	6.6	7.6	9.6	6.1	14.1	10.5
S	71.0	186	7.2	8.1	13.9	5.7	6.2	9.1	8.5	5.0	12.1	11.2
Mean	67.8	178	8.2	7.6	12.4	5.5	6.0	8.5	9.2	5.6	12.7	11.8

* TC = tetracycline; OTC = oxytetracycline; CTC = chlortetracycline; DMCT = demethylchlortetracycline. Each was given in a dose (labeled) of 500 mg., except for the 250 mg. dose designated $DMCT_{250}$.

The nonrenal removal rate (K_{nr}) was expressed in per cent per hour:

$$K_{nr} = K_s - K_r.$$

Plasma binding. Dialysis equilibrium studies were performed twice with each of the four analogues. The hydrochloride was used both times except for OTC, of which the base was employed on the first occasion. A 0.1 per cent aqueous solution of each drug was diluted in Krebs-Ringer phosphate buffer, pH 7.4, to the desired concentrations, namely 5, 12 and 20 μg. per ml. Five ml. amounts of normal human plasma were placed in cellophane bags (Nojax Casing No. 24, Visking Corporation), which were sealed and placed, in duplicate, each into a sterile screw-capped tube containing 5 ml. of antibiotic diluted in the Krebs-Ringer phosphate buffer. The tubes were placed in a mechanical device at 4° C. and rocked 18 times per minute. The contents of one set of bag and bath fluids were harvested at 24 hours and those of the other at 48 hours and promptly stored at −20° C. until they were assayed. Before the assays, the specimens were diluted and adjusted, when necessary, to achieve uniform protein content and pH among the specimens and standard controls. Two sets of control tubes, in which buffer was substituted for plasma in the bag and 10 μg. of antibiotic per ml. placed in the bath, were run during the second experiment.

Plasma protein binding, expressed in per cent, was determined by the formula:

$$\text{Per cent bound} = \frac{\text{concentration in plasma} - \text{concentration in bath}}{\text{concentration in plasma}} \times 100.$$

RESULTS

Concentrations of antibiotics in serum

Figure 2 shows the levels of each antibiotic in serum at intervals after the intravenous injections of 500 mg. of the four tetracycline analogues and after the 250 mg. dose of DMCT for each subject; the mean values for all subjects are also charted. The initial rapid decline, representing distribution into various body compartments, was complete by two to three hours after the injection of each drug. Comparisons of the second or slower rate of decline indicate that the levels of CTC in the serum fell more rapidly than those of the other analogues; the levels of TC and OTC fell at intermediate rates, while the slowest decline was for both dose levels of DMCT. This was true of the values for each individual and of the mean for all four subjects.

Decay rates and half-lives of the antibiotics in the serum, calculated from these data, are given in Table II. CTC was removed from serum most rapidly, at an average rate of 12.4 per cent per hour; TC and OTC decayed at similar and intermediate rates, averaging 8.2 and 7.6 per cent per hour, respectively, and DMCT showed the slowest decay, namely, 6.0 and 5.5 per cent per hour after the 250 mg. and 500 mg. doses, respectively. Correspondingly, the shortest average half-life was 5.6 hours for CTC; the half-lives of TC and OTC were intermediate at 8.5 and 9.2 hours, respectively, while that of DMCT was the longest, namely, 11.8 and 12.7 hours after the 250 and 500 mg. doses, respectively. Although there were some variations among the different subjects in the half-life of these antibiotics, each analogue was found to have a distinct rate of decay in serum, within fairly narrow limits.

Relative distribution volumes

The calculated values for the RDV of the four analogues are given in Table III; these all show distributions greater than the total body weight, presumably due to sequestrations in certain organs such as the liver and reticuloendothelial system

TABLE III

*Relative volume distribution (RDV) of four tetracycline analogues after single intravenous injection**

Subject	RDV					Per cent RDV per Kg. body weight				
	TC	OTC	CTC	DMCT	$DMCT_{250}$	TC	OTC	CTC	DMCT	$DMCT_{250}$
	L.	*L.*	*L.*	*L.*	*L.*					
H	109.6	130.5	99.9	118.8	119.3	161	192	147	175	175
M	110.3	128.2	120.1	122.9	107.6	156	182	170	174	153
P	122.1	121.3	98.6	108.1	83.2	172	187	160	176	135
S	90.8	132.0	81.5	134.1	92.0	148	186	115	189	130
Mean	108.2	128.0	100.0	121.0	100.5	159	189	148	179	148

* See footnote, Table II.

(17–20). The largest RDV was that of OTC; this averaged 189 per cent of the body weight. The two doses of DMCT yielded different average values,[4] the larger (179 per cent for the 500 mg. dose) approximating that of OTC. The smaller dose of DMCT gave an average value similar to those calculated for TC and CTC, each being about 150 per cent of the body weight. There were considerable individual variations in the values among the subjects,[4] the smallest differences being noted with OTC.

Rates of clearance from serum

The rate of clearance of each of the four tetracycline anologues from serum, as calculated from the rate of decline of its concentration in serum and its RDV, is shown in Table IV. CTC was cleared at a much more rapid rate (more then 200 ml. per minute) than the other analogues and about twice as rapidly as DMCT; OTC and TC were cleared at similar rates that were intermediate between those of CTC and DMCT. The slightly more rapid decay of TC in serum in spite of its slower clearance from serum as compared with that of OTC (Table II) will be shown to be due to the more rapid renal clearance of the latter.

TABLE IV

*Clearance of four tetracycline analogues from serum after a single intravenous injection**

Subject	Clearance from serum				
	TC	OTC	CTC	DMCT	$DMCT_{250}$
	ml./min.	*ml./min.*	*ml./min.*	*ml./min.*	*ml./min.*
H	145	159	225	123	107
M	151	164	219	107	100
P	185	146	186	89	92
S	116	179	189	128	95
Mean	149	162	205	112	99

* See footnote, Table II.

[4] Presumably related in part, at least, to differences in time required for complete mixing and equilibration in the body compartments.

Renal clearances

The calculated renal clearances per 1.73 M.^{2} of body surface for each of the four tetracyclines and for creatinine are shown together with the ratio of these values in Table V. There were wide variations in the simultaneously performed creatinine clearances,[5] but each of the four tetracycline analogues was cleared at a fairly distinct rate, with only minor individual variations considering the small number of observations (12 for each mean value). OTC was the most rapidly cleared by the kidney (85 ± 14 per cent of the creatinine clearance); CTC and DMCT were each cleared at approximately the same rate, namely, about 30 per cent of that of creatinine, and TC was cleared

TABLE V

*Renal clearance of four tetracycline analogues compared with simultaneous creatinine clearance in four healthy young men**

Antibiotic	Renal clearance		$\frac{C_{ar}}{C_{creat}}$
	Antibiotic (C_{ar})	Creatinine (C_{creat})	
	ml./min./1.73M.^{2} ± S.D.		*% ± S.D.*
TC	73.5 ± 7.8	120.6 ± 21.3	62 ± 8
OTC	98.6 ± 5.7	115.6 ± 16.5	85 ± 14
CTC	32.1 ± 8.0	108.3 ± 8.7	30 ± 8
DMCT	35.2 ± 6.7	131.2 ± 41	27 ± 8
$DMCT_{250}$	35.3 ± 7.9	111.9 ± 41	32 ± 16

* See footnote, Table II.

[5] This was probably related to discrepancies in the timing of the urine collections. However, the means of creatinine clearances for any one individual were reproducible within much narrower limits.

at an intermediate rate, which was 62 ± 8 per cent of the creatinine clearance. The rate of renal clearance of each of the four analogues was independent of the rate of urine flow, as shown in Figure 3.

Urinary recoveries

The cumulative urinary excretion of each of the tetracycline analogues over a period of four days after the single intravenous dose is depicted in Figure 4. The per cent recovered was calculated on the basis of the assayed values of the administered doses, as shown in Table I. Almost all of the amounts excreted were recovered in the urine during the first 24 hours, but small amounts of each were still detected on the fourth day, except for CTC which could not be detected in urine after the third day. The most rapidly and completely excreted analogue was OTC; TC ranked next in these respects, followed by DMCT and CTC.

Figure 5 presents a comparison of the expected total urinary recovery of each analogue (as calculated from the ratio of the renal clearance to the clearance from serum) and the assayed amounts actually recovered. In each instance, the amount recovered was somewhat greater than the expected or calculated amount, but the corresponding values were in close agreement. The differences could have been due to either the errors in the assays, underestimation of the renal clearances or overestimations of the serum clearance rates.

The average per cent of the administered dose that was accounted for in the urine was: 70 per cent for OTC, 60 per cent for TC, 42 per cent for the two doses of DMCT and 18 per cent for CTC.

Rates of renal and nonrenal removal

The nonrenal removal rates, determined by subtracting the renal removal rates from the serum removal rates and expressed both in per cent removed per hour and as half-life, are presented in Table VI; the calculated renal removal rates, expressed in the same manner are also given. The latter rates, as expected, were more rapid for OT and OTC than for CTC and DMCT. Of par-

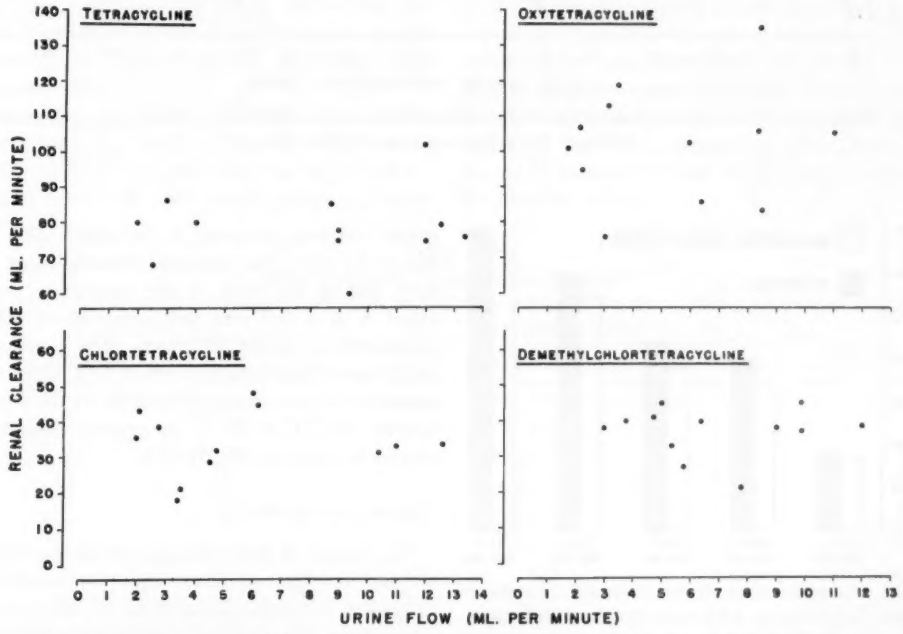

FIG. 3. CORRELATION OF RATES OF RENAL CLEARANCE OF FOUR TETRACYCLINE ANALOGUES AND RATES OF URINE FLOW

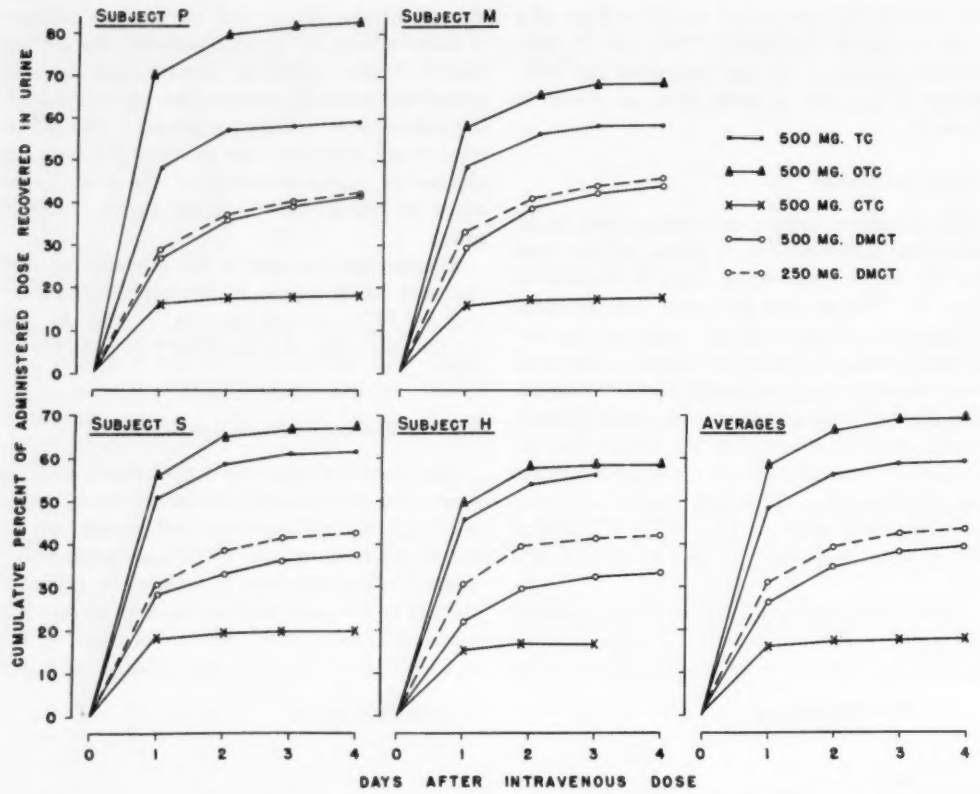

FIG. 4. CUMULATIVE PER CENT OF ADMINISTERED ANTIBIOTICS RECOVERED IN THE URINE AFTER SINGLE INTRAVENOUS DOSES OF FOUR TETRACYCLINE ANALOGUES

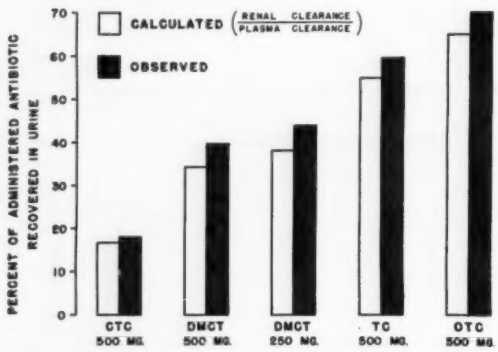

FIG. 5. COMPARISON OF ACTUAL URINARY RECOVERIES OF FOUR TETRACYCLINE ANALOGUES AND THE RECOVERIES EXPECTED FROM CALCULATIONS OF THE RATIO OF THE RENAL CLEARANCE TO THE SERUM CLEARANCE

ticular interest, however, is the observation that, except for CTC, the nonrenal removal rates were very similar for each of the analogues, namely about 3 to 4 per cent per hour, or at half-life equivalents of about 20 hours. The much more rapid rate of nonrenal removal of CTC thus represents the *in vivo* counterpart of the rapid decay in activity of CTC at 37° C. in aqueous solutions or plasma *in vitro* (6, 10, 20, 21).

Plasma protein binding

The results of determinations of plasma binding of the four tetracyclines done by equilibrium dialysis in two separate experiments and expressed as per cent of antibiotic bound after equilibration for 24 and 48 hours are presented in Table VII.

TABLE VI

*Calculated rates of removal of four tetracycline analogues by renal and nonrenal mechanisms after single intravenous injection**

Rates†	Subject	TC	OTC	CTC	DMCT	$DMCT_{150}$
K_r: Antibiotic removed by renal mechanisms %/*hr.*	H	4.8	3.9	2.9	2.0	2.7
	M	4.7	4.0	2.3	2.3	2.3
	P	5.1	5.5	2.1	2.4	2.7
	S	5.1	4.2	4.1	1.6	2.5
	Mean	4.9	4.4	2.9	2.1	2.6
$T_r/2$: Hours required to remove 50% of the antibiotic by renal mechanisms	H	14.3	17.6	23.9	34.7	25.7
	M	14.7	17.2	29.9	30.0	30.5
	P	13.6	12.7	32.5	28.8	25.2
	S	13.6	16.6	17.1	48.4	27.2
	Mean	14.1	16.0	25.9	35.5	27.2
K_{nr}: Antibiotic removed by nonrenal mechanisms %/*hr.*	H	3.1	3.4	10.6	4.2	2.7
	M	3.5	3.6	8.6	2.9	3.3
	P	4.0	1.8	9.0	2.5	3.9
	S	2.6	4.0	9.9	5.1	5.9
	Mean	3.3	3.2	9.5	3.4	4.0
$T_{nr}/2$: Hours required to remove 50% of the antibiotic by nonrenal mechanisms	H	22.5	20.6	6.5	16.5	25.9
	M	19.8	19.2	8.0	23.7	20.8
	P	17.4	38.7	7.7	27.4	17.9
	S	27.1	17.5	7.0	16.8	11.7
	Mean	21.7	24.0	7.3	20.1	19.1

* See footnote, Table II.
† See "Materials and Methods" for formulas from which these rates were calculated.

The initial concentrations in the baths were designed to provide levels of antibiotic in plasma comparable to those achieved with the usual therapeutic doses.

There were considerable differences in the per cent of each drug bound to plasma. This was to be expected from the errors inherent in the microbiological assay, the low concentration of drugs used and the considerable effects of changes in pH on tetracycline activity. In a control study in which buffer was substituted for plasma in the dialysis bag, the mean differences noted in either direction (*i.e.*, bag to bath or vice versa) was about 11 per cent, suggesting that these data should be interpreted with caution with respect to the absolute values.

TABLE VII

*Plasma binding of four tetracycline antibiotics**

Initial concentration in bath	Per cent bound to plasma							
	TC-HCl		OTC-Base	OYC-HCl	CTC-HCl		DMCT-HCl	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
μg./ml.				24 hours				
5	13	28	11	42		42	29	37
10	25	23	15	34	56	35	36	40
20	35	10	5	27	52	52	42	63
Mean	24	20	10	34	54	43	36	47
				48 hours				
5	30	20	12		49	36	34	38
10	24	25		17	30		57	23
20	30	28	10	28	46	66	52	42
Mean	28	24	11	23	42	51	48	34

* TC = tetracycline; OTC = oxytetracycline; CTC = chlortetracycline; DMCT = demethylchlortetracycline.

The data do indicate, however, that more of the CTC and DMCT were bound than TC and OTC. The mean per cent of antibiotic bound was 47 per cent for CTC and 41 per cent for DMCT (not significantly different), whereas an average of 24 per cent of TC and 20 per cent of OTC was bound to plasma (this difference, likewise, was not significant). The differences between the results obtained at 24 and 48 hours also are not significant. The greatest discrepancy, however, was in the results obtained with OTC in the two experiments, the base being used in the first and the hydrochloride in the second. The reason for this wide discrepancy is not clear since both samples were greatly diluted in phosphate buffer, pH 7.4.

DISCUSSION

The chemical structures of the four tetracycline analogues (Figure 1) differ from one another only in the presence or absence of either chlorine, or a hydroxyl or methyl group in one of the three positions on the "upper tier" of the molecule. These chemical differences are not accompanied by changes in the antibacterial spectrum, although differences in relative activity against certain organisms have been noted (5–12); they are, however, associated with major differences in the physical properties of the analogues. Thus, under physiological conditions of temperature and pH, CTC is rapidly inactivated, whereas the other analogues are fairly stable (1, 6, 21, 22); however, only DMCT remains stable at 100° C. and highly acid or alkaline pH. Other factors, such as differences in solubility and affinity for certain tissues, including blood plasma, may account for the different distribution, sequestration in certain organs and rates of elimination of these analogues.

Wide differences in the serum half-life of intravenously injected tetracyclines are reported, or can be calculated from data in the literature. The divergences are probably due, in large part, to the methods of assay and to the time allowed for equilibration in the body before the blood samples are drawn. In this laboratory, the twofold dilution method has generally yielded a shorter half-life than the cup-plate method (2, 23). Spitzy and Hitzenberger (24), employing a 2:3 serial dilution method for assay, reported the half-life of CTC, TC and OTC in blood following injection of 100 mg. to be 2.74, 2.04 and 0.73 hours, respectively. Calculations from data presented by others for TC show the half-life to be 3.0 to 6.6 hours after doses of 100 to 500 mg. (dilution method) (25), 7.5 hours after a 500 mg. dose (method unspecified) (26), and about 14 hours after a similar dose (dilution method) (27).

In the present study, in which the more accurate cup-plate method was used, the half-life of each analogue in serum was fairly distinct and was ranked in the same order for each subject. The longest mean half-life was that of DMCT (12.7 and 11.8 hours for the two doses), followed, in order, by those of OTC (9.2 hours), TC (8.5 hours) and CTC (5.6 hours). These values for DMCT and TC correspond to those previously reported after six hours' equilibration following oral doses, *viz.*, 11.8 and 8.2 hours, respectively (2), and are also in agreement with the value of about 10 hours for the serum half-life of DMCT calculated from the data of Sweeney, Hardy, Dornbush and Ruegsegger (3), the same assay method being used in all these studies.

Few reports are available on the relative distribution volume of the tetracycline analogues. Spitzy and Hitzenberger (24) reported the relative distribution volume of TC, CTC and OTC to be 95, 92 and 90 per cent of body weight, respectively. The values derived in the present study were considerably higher. OTC was found to have the largest distribution volume, exceeding 180 per cent of body weight in each subject with a mean value of 189 per cent. The 500 mg. dose of DMCT gave a mean value of 179 per cent followed by TC, CTC and the 250 mg. dose of DMCT at 159, 148 and 148 per cent of body weight, respectively. The reason for the discrepancy between the two DMCT values is not readily apparent, but may be due, in part, to differences in time required for equilibration. In any event, all these results must be interpreted with considerable caution because of *a*) the considerable errors involved in the microbiological assays of both the dose injected and the concentrations in the serum, and *b*) the error inherent in the single injection method of determining distribution volume due to incomplete or irregular equilibration of drug in body compartments (28). Nevertheless, the gross differences in distribution of OTC, TC and CTC may be important in interpretation

of data obtained in oral absorption studies in which these three drugs are compared (4). Because of its larger relative distribution volume OTC may be expected to produce lower blood levels than the other two analogues even if it were absorbed equally well. A better method of comparing the completeness of absorption may be the total urinary recovery and this is discussed below.

Although the relative distribution volumes of the tetracycline analogues are very large, the drugs are not evenly distributed throughout the body. Studies of the distribution of OTC and CTC in the tissues of the mouse by means of autofluorescence microscopy have shown the highest concentrations of these drugs to be in the liver, kidney and reticuloendothelial system (17, 18). Snell, Gorkuscha and Allen (19), employing C^{14}-labeled OTC in mice, found the greatest accumulation of radioactivity in the liver which also tended to retain drug in high concentrations even after the labeled drug could no longer be detected in the blood. Leevy, Zinke and Chey (20) using a similar method in man, also found OTC to be sequestered in the liver and slowly released into the blood.[6]

Apparently the size of the relative distribution volume is of no help in predicting the relative degree or order in which the analogues penetrate into the cerebrospinal fluid. Wood and Kipnis (29) obtained higher spinal fluid levels with TC than with OTC and CTC in man after comparable doses. In general, the penetration of these analogues into the cerebrospinal fluid is rather poor despite the large distribution volumes (30–32). Data on penetration of DMCT are not available. Differences in penetration into the cerebrospinal fluid may be an overriding consideration in the choice of drugs under certain conditions.

The major route of excretion of all of the tetracycline analogues is through the kidneys. The reported urinary recoveries of drug vary considerably, but generally TC and OTC have been recovered in larger amounts than CTC (26, 30–36). Sweeney, Hardy, Dornbush and Ruegsegger (3) recovered larger amounts of TC and less of CTC as compared with DMCT in an eight hour collection period after an oral dose. In the present study, the most complete recovery was obtained with OTC (70 per cent) followed, in order, by TC (60 per cent), DMCT (42 per cent) and CTC (18 per cent). These recoveries were in close agreement with the expected recovery of these drugs as calculated from the ratio of the rate of renal clearance to serum clearance (Figure 5).

Sirota and Saltzman (37) reported that CTC was cleared by the kidney at about 35 per cent of a simultaneously performed creatinine clearance and that its clearance rate was not altered by an osmotic diuresis. They inferred from this and from their studies on plasma protein binding that CTC was cleared by glomerular filtration. Similar conclusions can be drawn from the data for the four tetracycline analogues obtained in the present study. The analogue that was most rapidly cleared by the kidney was OTC (85 ± 14 per cent), followed in order, by TC (62 ± 8 per cent) and by CTC and DMCT, both of which were cleared at essentially the same rate, namely about 30 per cent of a simultaneous creatinine clearance. Variations in the rate of urine flow from 2 to 14 ml. per minute did not alter these clearances. These results are also in good agreement with those previously reported in an oral absorption study (2) (TC, 71 per cent and DMCT, 31 per cent of the simultaneous creatinine clearance, respectively).

It is realized that these results are based on observations in a small number of normal subjects and that there were considerable variations in some instances. Conceivably, patients with severe infections might handle these drugs differently (23).

It is reasonable to assume from the data presented on the rate and completeness of excretion of the tetracycline analogues into the urine that OTC would be the most readily and completely excreted of the analogues in the urine of patients with renal disease and TC only slightly less so. DMCT may be expected to be retained longer in the blood of a uremic patient, whereas CTC activity would be rapidly dissipated and very little would appear

[6] After submission of this paper it was shown that, following a single intravenous injection, DMCT, like the other three analogues, is concentrated in the liver and excreted in the bile where it is found in concentrations up to 32 times greater than in the simultaneously obtained serum. This would indicate that the higher and better sustained serum levels of DMCT cannot be explained by diminished excretion into the bile (41).

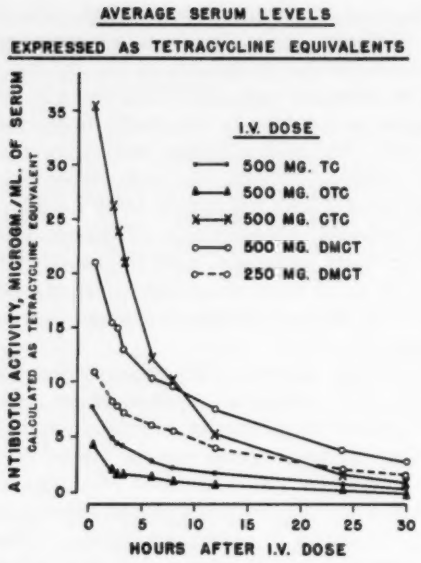

FIG. 6. AVERAGE CONCENTRATIONS OF ANTIBIOTIC ACTIVITY IN SERUM RESULTING FROM SINGLE INTRAVENOUS DOSES OF FOUR TETRACYCLINE ANTIBIOTICS, ALL CALCULATED AS TETRACYCLINE ACTIVITY AND PLOTTED ON AN ARITHMETIC SCALE

in the urine. These expectations are supported in part by calculations of the rate of nonrenal removal of the analogues. Each was found to be removed at about 3 to 4 per cent per hour with the exception of CTC, of which 9.5 per cent was removed per hour by nonrenal mechanisms. Thus, despite the fact that CTC and DMCT are cleared by the kidney at about the same rate, the serum half-life of CTC is about one-half that of DMCT and less than one-half as much is recovered in the urine.

The data pertaining to the rate of nonrenal removal of TC is somewhat at variance with those reported in an earlier study on the persistence of TC in the blood of anuric patients (23). In that study TC was removed from the serum of anuric patients at a rate of 0.6 to 1.2 per cent per hour as compared with a mean rate of 3.3 per cent per hour calculated in the present study. These differences may be due to *a*) the shorter period of equilibration in the present study, *b*) differences between assay by the twofold dilution method and the cup-plate method and *c*) differences in the "metabolism" of the drug in normal and uremic patients. On the other hand, there is fairly good agreement between the nonrenal removal rate of 9.5 per cent per hour obtained in the present study for CTC and the corresponding rate of about 6 to 10 per cent per hour in uremic patients in the earlier study (23).[7]

Sirota and Saltzman (37) using a fluorophotometric method of assay and somewhat higher concentrations of antibiotic than those employed here, found 65 to 70 per cent of CTC bound to human plasma proteins (mainly albumin), which is greater than the binding demonstrated here.[7] In the present study, CTC and DMCT were found to be bound to plasma to a similar extent, namely 41 and 47 per cent, respectively, and to about twice the extent of the binding of OTC and TC (20 and 24 per cent, respectively).[8]

In previous studies from this laboratory (2, 4, 38–40), dealing with blood levels of related antibiotics that are potentially of clinical interest, emphasis has been placed on the importance of either using a single standard of comparison of activity or of comparing the activity of the serums against a variety of test organisms. In the present study, the value for the concentration of each antibiotic in serum and urine was expressed in terms of its own standard since interest here was focused primarily on comparative distribution in the body and relative rates of absorption and excretion which are adequately reflected in this manner. From the clinical point of view, however, comparisons of relative activity of serum or other body fluids at different times after comparable doses are important, and these are best obtained by calculating the levels from the same reference standard.

Such a comparison for the average levels in serum after the single intravenous doses of the four tetracycline analogues is shown in Figure 6, in which tetracycline is used as the standard of

[7] The difference may represent bound antibiotic that is fluorescent but not antimicrobially active.

[8] Wozniak (42), in more thorough dialysis equilibrium studies under physiological conditions of temperature and pH, found that the degree of binding by dog and human plasma for drug concentrations ranging from 1 to 10 μg. per ml. was approximately twice as much for CTC (62 per cent) as for TC (34 per cent) and was intermediate (49 per cent) for DMCT. She also demonstrated that the binding of tetracycline increased with increasing alkalinity over the pH range of 6.7 to 8.1.

reference and the levels of activity are plotted on an arithmetic scale. Similar relationships would be revealed with the use of any of the other three analogues as a standard of reference, except that the absolute values would be altered depending on which of the drugs was used. The considerably greater levels of activity produced in serum by DMCT as compared with either TC or OTC are readily apparent in this figure. Indeed, the 250 mg. dose of DMCT yielded greater activity throughout the period of observation than the 500 mg. dose of either TC or OTC. CTC, on the other hand, produced much greater activity than the same dose of DMCT during the first eight hours, after which the levels from the latter were greater.

It is to be borne in mind, in addition, that these relationships also vary with the test organism. Thus, against the strain of *Staphylococcus aureus* ATCC 6539P used in the turbidimetric assay of the intravenous preparations that were administered, DMCT was about 90 per cent as active as CTC, three times as active as TC and 1.9 times as active as OTC. Against the test strain of *B. cereus* used in the assays of the serums, DMCT was 70 per cent as active as CTC, three times as active as TC and four times as active as OTC.

SUMMARY AND CONCLUSIONS

The distribution and excretion of the four tetracycline analogues—tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and demethylchlortetracycline (DMCT)—were studied following single intravenous injections in four healthy young men. Each drug was given in a dose of 500 mg. and an additional dose of 250 mg. of DMCT was also studied. Estimations of the binding of these analogues to plasma proteins was determined by equilibrium dialysis *in vitro*.

The mean half-life in serum was longest for DMCT, shortest for CTC and intermediate for TC and OTC.

The greatest mean relative volume of distribution was that of OTC, but all four analogues were distributed through a relative volume greater than the total body weight, indicating that there is considerable sequestration in body compartments, notably the liver.

DMCT was cleared from serum, on the average, about one-half as rapidly as CTC; TC and OTC were cleared at similar rates which were intermediate.

The renal clearances of DMCT and CTC were similar and averaged about 30 per cent of the simultaneous clearance of creatinine; the corresponding values for TC and OTC were 62 and 85 per cent, respectively. The renal clearance rates were not significantly affected by changes in urine flow from 2 to 14 ml. per minute. All of these drugs are probably cleared by glomerular filtration.

The average 96 hour urinary recovery of OTC was 70 per cent of the administered dose, as compared with 60 per cent for TC, 42 per cent for DMCT and only 18 per cent for CTC. These values corresponded closely to those calculated from the ratio of renal clearance to serum clearance. The greatest proportion of the total of each drug excreted in the urine was recovered in the first 24 hours.

The nonrenal removal rates of TC, OTC and DMCT were very similar and averaged 3 to 4 per cent per hour; CTC, however, was removed much more rapidly by nonrenal mechanisms (9.5 per cent per hour).

Both DMCT and CTC were bound to plasma to about the same extent (41 and 47 per cent, respectively) and to about twice the extent of OTC and TC (20 and 24 per cent, respectively), but there were wide variations in these results.

Of the four analogues, therefore, DMCT clearly produces the most sustained levels in the blood, OTC is the most completely excreted in the urine, TC produces slightly less sustained levels in the blood and slightly lower urinary recoveries than OTC, and CTC yields the least sustained levels in blood and the smallest recovery in the urine.

The importance of using a single standard of reference in comparing activity of related drugs was discussed and illustrated by the levels obtained in the serums in the present study.

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ADJUSTMENT TO ALDOSTERONE OR DESOXYCORTICOSTERONE ACETATE INDUCED SODIUM RETENTION IN PATIENTS WITH ADDISON'S DISEASE*

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Long term administration of aldosterone (1) or desoxycorticosterone acetate (DCA) (2) to normal subjects has been shown, in certain instances, to produce continued renal potassium loss but only transient sodium and chloride retention. This adjustment of the renal response to sodium-retaining adrenal steroids has been termed an "escape" (3) from the expected effects of these compounds, and is seen clinically in the absence of edema with primary aldosteronism. The mechanism of this "escape" from sodium retention, that occurs despite continued excess of steroid sodium-retaining substances, is unknown. Some findings have suggested that the adrenal gland may play a role by producing a substance which either promotes sodium excretion or inhibits the sodium-retaining effect of other hormones.

An adrenal cortical "salt-losing hormone" was postulated by Wilkins and Lewis (4) and Jailer (5) to explain renal salt loss in cases of congenital adrenal hyperplasia. Rosemberg (6) suggested that this might be explained by an aldosterone-inhibiting effect of certain 21-desoxypregnane steroids, and Klein and co-workers (7) reported that partially purified material from urine of patients with congenital adrenal hyperplasia caused increased urinary sodium excretion in rats. Recently, Neher, Meystre and Wettstein (8) reported the isolation of 3α,16α-dihydroxy-pregnan-20-one from patients with the adrenogenital salt-losing syndrome and demonstrated a salt-losing potential of this steroid in adrenalectomized rats. Increased renal sodium excretion has been related to several other steroid compounds as well. Thorn and co-workers (9) reported that patients with Addison's disease who conserved sodium, while receiving 5 mg. of DCA per day, showed an immediate increase in sodium excretion when 100 mg. of cortisone acetate per day was added. Progesterone has been shown by Landau and associates (10, 11) to result in natriuresis in normal subjects and treated patients with Addison's disease, and by Kagawa (12) to block the sodium-retaining effect of DCA in rats. Johnson (13) and Axelrod, Cates, Johnson and Luetscher (14) found that certain 11-oxycorticosteroids and 11-desoxycorticosteroids produced increased sodium excretion in water-loaded, adrenalectomized rats. A synthetic steroid-17-spirolactone, 3-(3-oxo-17β-hydroxy-4-androsten-17α-γ^1)-propionic acid α-lactone, and its 19-"nor" analog, counteract the sodium-retaining properties of DCA and aldosterone, possibly as a competitive inhibitor (15, 16). Neher and co-workers (17) have isolated another compound, 3β,16α-dihydroxy-allopregnan-20-one, from hog adrenals which, under certain biological conditions, also acts as a sodium-excreting factor.

Additional interest in the role of the adrenal gland in the "escape" was stimulated by the reports of severe edema in some patients with Addison's disease treated solely with DCA (18–23), in contrast to the response of normal subjects (1, 2). Also, in canine studies, Kovach and associates (24) reported that adrenalectomy prevented an expected increase in sodium excretion following intracarotid injection of hypertonic sodium chloride.

In the light of these observations, the following study of the "escape" mechanism in patients with Addison's disease given large amounts of aldosterone or DCA was undertaken. The data indicate that an adjustment to aldosterone or DCA

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induced sodium retention may occur in the absence of normal adrenal function.

MATERIAL AND METHODS

The five subjects of this study were three patients with Addison's disease of unknown etiology and two who had been completely adrenalectomized because of Cushing's syndrome with bilateral adrenal hyperplasia. The diagnosis of Addison's disease had been established clinically by low excretion of 17-ketosteroids (25) and 17-hydroxycorticosteroids (26), and by the absence of increased excretion of these steroids during administration of 25 units of adrenocorticotropic hormone (ACTH) intravenously over eight hours for two or three consecutive days. All had been maintained in good health with both cortisone and DCA or fluorohydrocortisone maintenance therapy, and were hospitalized on the Metabolic Ward for purposes of this study.

The subjects were given constant diets throughout the study. Body weight was determined under standard conditions at the beginning of each metabolic day. Urine was collected and stored at 5° C. Measurements were made of sodium and potassium by flame photometry with an internal standard and of creatinine by the Jaffé reaction following precipitation with Lloyd's reagent.

The aldosterone used in this study was synthetic *d,l*-aldosterone-21-monoacetate which has approximately 50 per cent of the activity of the natural product (kindly provided by Drs. Robert Gaunt and C. H. Sullivan, Ciba Pharmaceutical Products, Inc., Summit, N. J.). The authenticity of this material was tested by paper chromatography before and after enzymatic hydrolysis. Recovery of steroid with the characteristics of aldosterone was consistent with a pure compound with the exception that a small amount of the aldosterone was initially not acetylated. It was administered intramuscularly in sesame oil in divided doses at eight hour intervals. DCA was administered intramuscularly every 12 hours. Vasopressin (Pitressin®) was given to one patient subcutaneously in aqueous suspension every eight hours.

Although endogenous adrenal corticosteroid secretions were not a factor in these studies, cortisone given as maintenance therapy could not be completely eliminated. The studies were designed with variations in cortisone dosage to eliminate its importance to a degree compatible with the health of the subjects.

PROCEDURE AND RESULTS

Study I: Aldosterone with continued cortisone therapy (Figure 1, Table I)

W.D., a 64 year old male, was given 3 mg. of *d,l*-aldosterone monoacetate while being maintained with 50 mg. of cortisone daily. Sodium intake was 174 mEq., chloride, 182 mEq. and potassium,

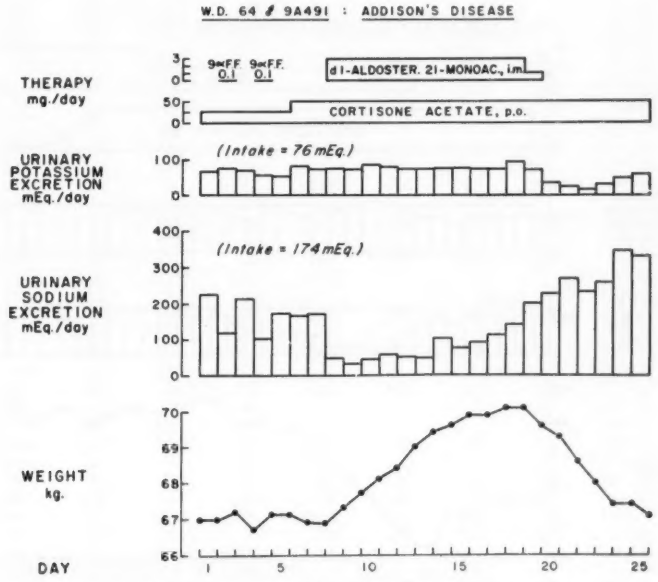

FIG. 1. ALDOSTERONE WITH CONTINUED CORTISONE THERAPY

The "escape" from renal sodium excretion began on the seventh day of aldosterone administration.

TABLE I

Metabolic data on Subject W.D.

Day	Body wt.	Urine					Blood			Blood pressure	Therapy		
		Volume	Na	K	Cl	C_{cr}*	Na	K	Cl		Cortisone	F.F.†	Aldosterone‡
	Kg.	*ml./da.*		*mEq./da.*		*ml./min.*		*mEq./L.*		*mm.*		*mg./da.*	
1	67.0	1,740	226	68	207	113	146	4.5	103	116/64	25		
2	67.0	1,220	119	78	145					120/70	25	0.1	
3	67.2	2,110	213	71	222	128	142	4.8	111	124/64	25		
4	66.7	1,250	103	57	136					120/74	25	0.1	
5	67.1	1,880	171	56	188	124	145	4.3	107	124/68	25		
6	67.1	1,400	166	82	178					122/72	50		
7	66.9	2,050	172	75	178	115	140	4.1	107	122/76	50		
8	66.9	960	48	76	103	140	138	3.9	111	118/62	50		3
9	67.3	1,320	32	73	79	121	140	4.1	108	132/72	50		3
10	67.7	1,420	45	86	85	133	141	3.8	113	140/76	50		3
11	68.1	1,360	58	80	106	125	144	4.0	111	164/88	50		3
12	68.4	800	51	75	82	136	146	3.6	109	160/88	50		3
13	69.0	1,020	47	76	97	141	139	3.9	107	144/80	50		3
14	69.4	1,820	101	76	155	132	135	3.9	110	148/80	50		3
15	69.6	1,520	75	77	132	129	134	3.9	109	160/84	50		3
16	69.9	1,160	91	75	132	149	137	3.5	114	164/90	50		3
17	69.9	1,560	111	75	164	130	136	3.8	111	180/90	50		3
18	70.1	2,030	138	114	165	127	143	3.6	113	168/80	50		1
19	70.1	2,120	200	73	205	138	141	3.7	113	170/82	50		
20	69.6	2,140	226	37	214	136	148	3.9	111	160/76	50		
21	69.3	2,200	268	26	260	121	142	3.6	109	158/78	50		
22	68.6	1,680	230	18	213	127	142	3.8	106	148/82	50		
23	68.0	2,020	259	31	240	118	138	4.1	101	150/78	50		
24	67.7	1,900	346	47	270	125	138	3.9	114	154/76	50		
25	67.4	1,780	332	59	297	112	138	3.5	108	140/72	50		

* Corrected to 1.73 $M.^2$.
† 9-α-Fluorohydrocortisone.
‡ *d, l*-Aldosterone-21-monoacetate.

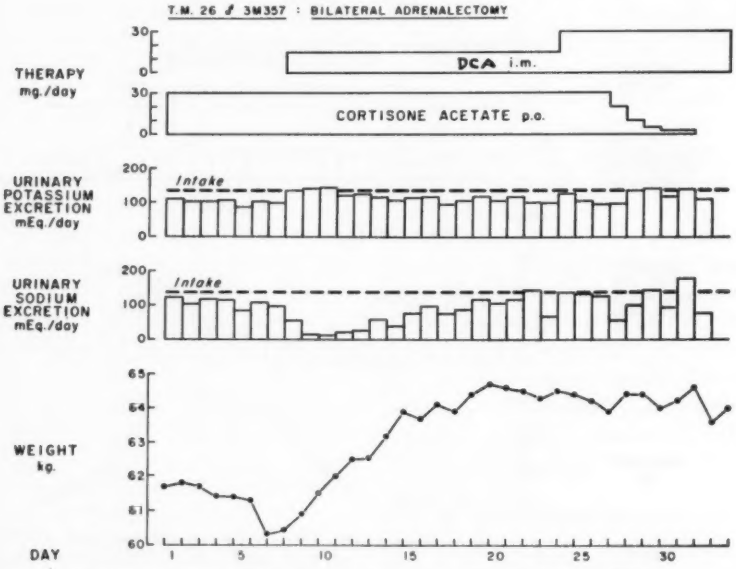

FIG. 2. CORTISONE WITHDRAWAL AFTER THE "ESCAPE" FROM DCA

Urinary sodium excretion was not significantly affected by doubling the amount of DCA administered or by gradual reduction of cortisone.

76 mEq. daily. Prior to the administration of aldosterone, sodium retention alternated with increased sodium excretion as the result of 0.1 mg. of 9-α-fluorohydrocortisone given every other day. When aldosterone was administered, sodium excretion promptly diminished and remained between 32 and 58 mEq. per day for six days, accompanied by rapid weight gain. Chloride retention also occurred. Following a weight increase of 2.5 Kg., sodium chloride excretion gradually increased and the rate of weight gain diminished despite continued aldosterone administration. A moderate elevation of blood pressure persisted during the period of increased weight. After omission of aldosterone, there was potassium retention, increased sodium and chloride excretion and a loss of weight. There were no significant variations in endogenous creatinine clearance.

Study II: Cortisone withdrawal after "escape" from DCA (Figure 2, Table II)

In this study, the mineralocorticoid was increased and the glucocorticoid decreased after the "escape" from sodium chloride retention had occurred. T.M., a bilaterally adrenalectomized male, 26 years of age, received 136 mEq. of sodium, 145 mEq. of chloride and 138 mEq. of potassium daily. Maintenance hydrocortisone, 30 mg. daily, was given initially. During the first seven days of DCA administration, sodium excretion was 12 to 56 mEq. per day and there was a weight gain of 3.5 Kg. Thereafter, sodium excretion was as great as before DCA and there was no further consistent weight gain. The daily excretion of sodium following the "escape" was not significantly affected by doubling the amount of DCA

TABLE II

Metabolic data on Subject T.M.

Day	Body wt.	Urine					Blood			Blood pressure	Therapy	
		Volume	Na	K	Cl	C_{cr}*	Na	K	Cl		DCA	Cortisone
	Kg.	*ml./da.*	*mEq./da.*			*ml./min.*	*mEq./L.*			*mm.*	*mg./da.*	
1	61.7	1,850	121	110	134					122/80		30
2	61.8	1,280	102	102	115	107	140	5.0	108	124/80		30
3	61.7	2,000	118	102	126					120/80		30
4	61.4	1,700	117	105	132	117	143	4.9	106	124/70		30
5	61.4	1,330	83	87	106					118/80		30
6	61.3	1,940	108	101	121	124	138	4.9	104	110/60		30
7	60.3	1,540	96	99	115					120/72		30
8	60.5	2,080	55	133	74					112/80	15	30
9	60.9	1,080	14	140	30	136	139	5.1	105	116/74	15	30
10	61.5	1,260	12	141	29					114/65	15	30
11	62.0	1,420	21	120	56	115	136	4.5	109	100/60	15	30
12	62.5	1,400	26	122	63					92/56	15	30
13	62.6	1,380	58	113	99	146	135	3.9	110	110/60	15	30
14	63.4	1,140	40	104	52					130/70	15	30
15	63.9	1,440	77	112	82	123	139	4.1	111	120/60	15	30
16	63.7	1,240	96	113	117					110/60	15	30
17	64.1	1,120	76	92	92					126/72	15	30
18	63.9	1,300	86	103	100	125	144	4.4	109	132/80	15	30
19	64.4	1,400	115	116	132					124/80	15	30
20	64.7	1,260	104	104	130	136	141	4.0	107	140/90	15	30
21	64.6	1,560	115	115	128					134/90	15	30
22	64.5	1,590	142	99	160	134	142	4.4	108	130/84	15	30
23	64.3	1,180	69	98	82					127/74	15	30
24	64.5	1,360	136	123	147					140/92	30	30
25	64.4	1,560	132	102	162	120	144	3.9	105	142/94	30	30
26	64.2	1,640	123	92	138					136/84	30	30
27	64.9	980	54	93	82	141	138	3.8	106	140/80	30	20
28	64.4	1,600	100	131	123	138	142	3.8	109	142/84	30	10
29	64.4	1,980	143	138	161	116	134	3.9	105	138/80	30	5
30	64.0	1,300	52	92	68	128	147	4.3	110	122/80	30	2.5
31	64.2	1,490	92	115	103					130/76	30	2.5
32	64.6	2,400	178	137	152					144/80	30	2.5
33	63.6	1,620	85	107	99	139	135	3.8	108	120/74	30	
34	64.0	1,460	93	111	117	124	139	3.6	108	132/68	30	

* Corrected to 1.73 $M.^2$.

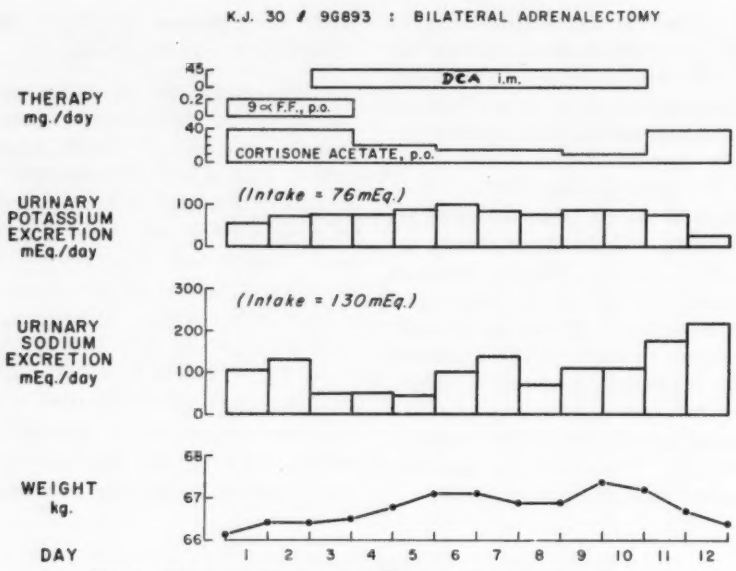

FIG. 3. CORTISONE WITHDRAWAL DURING "ESCAPE" FROM DCA

administered to 30 mg. daily, by gradual reduction of hydrocortisone, nor by total omission of hydrocortisone for one day. Endogenous creatinine clearance and urine volume were not significantly affected by the withdrawal of hydrocortisone. The observations could not be continued, however, due to a rapid decrease in the subject's sense of well-being when hydrocortisone was withdrawn.

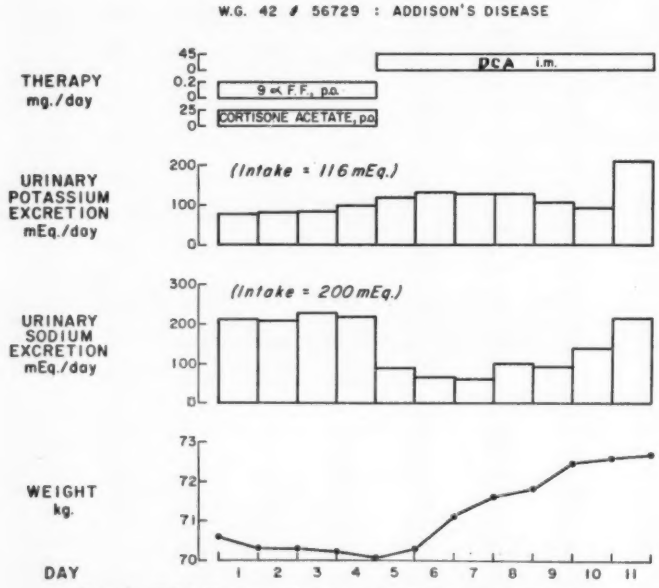

FIG. 4. DCA DURING COMPLETE CORTISONE WITHDRAWAL

The "escape" was conclusive on the sixth and seventh day of DCA administration.

Study III: Cortisone withdrawal during "escape" from DCA (Figure 3)

Another bilaterally adrenalectomized male, age 30, was given 45 mg. of DCA daily for nine days while cortisone was gradually diminished from 37.5 to 10 mg. daily. Sodium intake was 130 mEq. and potassium, 76 mEq. daily. Sodium excretion fell to 49 mEq. on the first day of DCA given in conjunction with 0.2 mg. of 9-α-fluorohydrocortisone, the maintenance level of mineralocorticoid in this case. On the fourth and fifth days of DCA, when only 15 mg. of cortisone was being given, sodium excretion increased to 100 and 137 mEq. per day. Thereafter a cyclic pattern of sodium excretion, previously seen during the "escape," continued despite further reduction of the cortisone dosage to 10 mg. per day. It is noteworthy that the "escape" occurred in this case after a weight gain of only 0.7 Kg. as compared to a weight gain of 2.5 to 3.0 Kg., as seen in normal subjects (1) and the other patients in this study. A probable explanation for this finding is that excess body sodium and water were already present when DCA was first given, due to daily use of 0.2 mg. of 9-α-fluorohydrocortisone as maintenance therapy. This explanation is supported by other studies which showed that the sodium-retaining effects of 9-α-fluorohydrocortisone were inversely related to the initial exchangeable sodium in patients with Addison's disease (27). Moreover, it has been observed that patients with Addison's disease, who are depleted of salt and water, gain weight in excess of 3 Kg. before achieving sodium balance when given large amounts of DCA.

Study IV: DCA during complete cortisone withdrawal (Figure 4)

Cortisone was completely withdrawn while the patient with Addison's disease, age 42, was given 45 mg. of DCA daily. Previous maintenance therapy had been cortisone, 25 mg. and 9-α-fluorohydrocortisone, 0.2 mg. daily. The diet in this study provided 200 mEq. of sodium and 116 mEq. of potassium per day. Sodium excretion was lowest, 62 mEq., on the third day of DCA administration and the "escape" was definite on the sixth day, after a weight gain of 2.3 Kg., when 142 mEq. of sodium was excreted. On the last day of DCA, 214 mEq. of sodium was excreted.

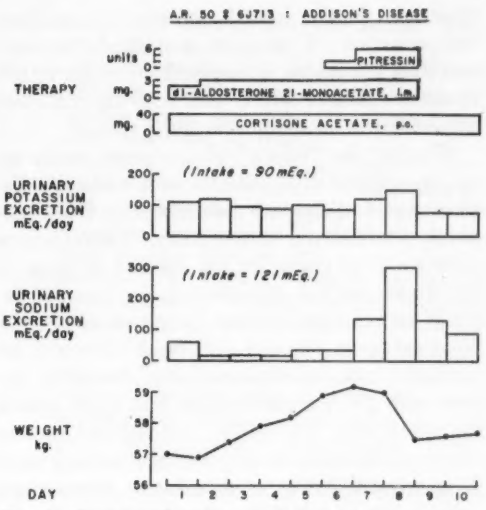

FIG. 5. PITRESSIN® DURING ALDOSTERONE AND CORTISONE ADMINISTRATION

Marked natriuresis and diuresis occurred when Pitressin® was given after five days of aldosterone administration.

Study V: Pitressin® during aldosterone and cortisone therapy (Figure 5)

A 54 year old woman with Addison's disease, while receiving 40 mg. of cortisone and 3 mg. of *d,l*-aldosterone acetate daily, was given aqueous Pitressin®. Sodium intake was 121 mEq. and potassium, 90 mEq. per day. Following a weight gain of 2 Kg., marked natriureses of 134 and 301 mEq. and diuresis occurred on the second and third days of Pitressin® administration despite continued aldosterone. It may be noted that neither weight gain nor water retention was apparent during the 24 hour periods of Pitressin® administration.

DISCUSSION

It was previously shown, in normal subjects receiving 3 to 6 mg. of *d,l*-aldosterone-21-monoacetate daily, that sodium and water retention ceased following weight gain of approximately 3 Kg. (1). Since the responses of the patients with Addison's disease in this study appear similar to

those seen in the normal subjects, it is unlikely that secretion of an adrenal cortical "sodium-excreting" hormone is responsible for the return to sodium balance which occurs during continued administration of sodium-retaining steroid.

Whether the "escape" phenomenon would occur or continue in all patients with Addison's disease treated solely with aldosterone or DCA cannot be determined from this study. Diminution or withdrawal of glucocorticoid therapy in three of the studies did not indicate that the "escape" depends on cortisone but the period of withdrawal from cortisone was not prolonged. Reports describing treatment of patients with Addison's disease with DCA alone indicate that many gained not more than 2 to 4 Kg. and did not become severely edematous despite administration of up to 40 mg. of DCA daily combined with large sodium chloride intake (28–32). However, several cases of massive edema and congestive heart failure have been attributed to DCA therapy (18–23). The number of complicating factors which may have prevailed make the interpretation of these cases difficult. Pre-existing cardiac or renal disease may have been present in some (19, 21, 23), and others were severely ill due to adrenal insufficiency. In this respect, it should be noted that continued sodium retention is not the normal response to a mineralocorticoid, and that patients with congestive heart failure, cirrhosis with ascites, or nephrosis, respond differently from normal subjects (33). These patients show virtually complete sodium retention, continuous weight gain without an "escape" and urinary potassium excretion not in excess of intake. Patients with pre-existing abnormal sodium retention and edema, or patients with low cardiac or hepatic reserve with a tendency to edema formation, would not be expected to "escape" from the sodium-retaining effects of aldosterone or other mineralocorticoids. It may be possible that severe, untreated glucocorticoid deficiency would result in abnormal renal function and continued sodium chloride retention during treatment with a mineralocorticoid. This is not to say, however, that an adrenal hormone is directly responsible for the "escape" phenomenon.

SUMMARY

Five patients with Addison's disease, in two due to bilateral adrenalectomy, received large amounts of aldosterone or desoxycorticosterone acetate while on constant diets. Glucocorticoid therapy was varied in the different patients as follows. It was held constant, gradually reduced after the "escape" from sodium retention, gradually reduced before and during the "escape," and completely omitted. In one study, Pitressin® was given with aldosterone. In each of these patients with Addison's disease, sodium chloride retention was transient and apparently was not acutely affected by change in glucocorticoid therapy. It is concluded that an adrenal cortical "sodium-excreting" hormone is not responsible for the "escape" from sodium retention that occurs despite continued administration of sodium-retaining hormone.

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NEGATIVE PRESSURE RESPIRATION, WATER DIURESIS AND NATRIURESIS IN NORMOTENSIVE, HYPERTENSIVE AND PREHYDRATED NORMOTENSIVE SUBJECTS *

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Sieker, Gauer and Henry (1), examining normotensive subjects, have demonstrated that breathing against continuous negative pressure elicits a moderate water diuresis, believed to be mediated by stretch receptors in the left atrium and reflex inhibition of the secretion of the antidiuretic hormone. This water diuresis, which has been confirmed by others (2–4) is not accompanied in normotensive subjects by any noteworthy increase in the excretion of sodium. On the other hand, the lability of sodium excretion in subjects with essential hypertension is well known. Effective natriuretic stimuli include urethral catheterization (5) and the infusion of mannitol (6), saline (7–11) or glucose and the ingestion of water or beer (12). It therefore seemed of interest to determine whether or not negative pressure breathing produces natriuresis in hypertensive subjects; and since, as Ladd (13) has shown, massive prehydration of normotensive subjects predisposes to excessive natriuresis and water diuresis during subsequent saline infusion, it was of equal interest to determine how prehydrated subjects would react to negative pressure breathing.

METHODS

Thirty-two observations have been made on 31 subjects, eight males and 23 females, ranging in age from 19 to 59 years, selected from the wards and Hypertension Clinic of the Third (New York University) Medical Division of Bellevue Hospital. Essential hypertension (without evidence of circulatory failure or advanced renal impairment) was present in 11 subjects; the remaining 20 were normotensive and free of cardiovascular renal disease. All were maintained on the regular hospital diet except five hypertensive subjects from the outpatient clinic who received a home diet unrestricted in respect to sodium intake.

Negative pressure breathing (NPB) of room air was accomplished by means of a standard mouthpiece and nasal clamp, and respiration to and from an 80 L. reinforced polyethylene cylinder (garbage container) which was partially evacuated by a high flow vacuum cleaner. The pressure was maintained close to -15 cm. water, as measured with a water manometer, by adjustment of an air intake valve. All subjects were instructed in the mechanics of the test beforehand and assured that it was harmless. Negative pressure of -15 cm. water proved tolerable for all subjects, but in four subjects a pressure of -25 cm. water proved to be intolerable because of the effort required.

Previous investigators have studied subjects who were moderately hydrated, but because of the lability of sodium excretion in hypertensive subjects we have avoided both water and saline and carried out our observations in the hydropenic state attained by 12 to 15 hours' abstinence from fluids. Because of this circumstance, urine was collected by catheter, with the utmost care with respect to emptying the bladder and expulsion of residual urine by means of air. Special precautions were taken to have the basal urine flow at as constant a level as possible.

In both control and hypertensive subjects, the test was started at 8:00 a.m., after a one to two hour ambulatory period. With the subject in a supine position (one pillow), the bladder was catheterized, emptied and the catheter left in place. One to two hours more were allowed to elapse after catheterization to avoid postural diuresis. At this time the urine flow had become fairly constant, as judged by the fact that in three ensuing quantitative urine collection periods of 15 minutes each (hereafter designated as basal) the rate of flow in two successive periods did not differ, at most, by more than 25 per cent. NPB was started at the end of the third basal collection and continued for 30 minutes; a fourth urine sample was collected at the end of 15 minutes without interruption of NPB, and a fifth immediately after the cessation of NPB. Venous blood samples were drawn at the beginning and end of the basal collection periods, shortly after the cessation of NPB and again at the end of the test. Urine was preserved in stoppered Lusteroid tubes and blood was centrifuged at once and the plasma stored in stoppered tubes. Heparin, two drops per 15 ml. of blood, was used as an anticoagulant.

The preparatory protocol for prehydrated subjects differed necessarily from that described above. They were taken off fluids 12 to 15 hours before coming to the Research Unit at 8:00 a.m. Here they drank 2 L. of tap

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water in the course of 20 to 40 minutes and remained ambulatory thereafter for five to six hours, after which they assumed the supine position and were catheterized. They were allowed to eat a chocolate bar during the ambulatory period, but no fluids were permitted after prehydration. The first basal urine collection period was begun after two to three hours in the supine position when the urine flow had become fairly constant, and well after the last phase of the water diuresis and presumably beyond the end of any postural diuresis. After three urine collection periods of 15 minutest each, NPB was instituted (some eight to nine hours after water loading) for 30 minutes as described above, followed by three post-NPB urine collection periods.

Osmolality of plasma and urine was determined by means of a Johlin freezing point apparatus (14) and the thermistor and bridge-null-point-detector unit of Bowman, Trantham and Caulfield (15). Sodium was determined by flame photometry, employing lithium as an internal standard. Inulin clearances were avoided because even a small infusion may elicit natriuresis in hypertensive subjects (10).

Because the water diuresis induced by NPB is transient, we have arbitrarily chosen as representing the maximal response to NPB that urine period which showed the greatest value of V; this was either the last period of NPB or the period immediately following. In half the subjects of each group, urine flow was maximal in the last period of NPB and in the remaining half, urine flow was maximal in the period immediately following NPB, but in either case this is the period designated NPB in Tables I, II and III.

Values for urine volume (V), sodium excretion ($U_{Na}V$), total solute excretion ($U_{osm}V$), osmolal clearance (C_{osm}) and free water clearance (C_{H_2O}), observed during (or immediately after) NPB have been compared with basal values in each subject by Wilcoxon's signed-rank test for matched pairs (16, 17), a nonparametric method of statistical analysis. Nonparametric analysis seemed more appropriate than the parametric method (which employs the mean and standard deviation) because it can be applied to small samples which may not have a Gaussian distribution, and its sensitivity is nearly as great as that of the "t" test, applied to samples with a Gaussian distribution which *a priori* assumes independent variation among the variables under consideration. However, for comparison the same data have been analyzed by the parametric method (16), and

TABLE I

*Normal hydropenic subjects: Response to negative pressure breathing**

Subject	Body surface area	Procedure	V	U_{Na}	$U_{Na}V$	U_{osm}	$U_{osm}V$	C_{osm}	C_{H_2O}	P_{Na}	P_{osm}
	M.²		*ml./min.*	*mEq./L.*	*μEq./min.*	*mOsm./Kg. H_2O*	*μOsm./min.*	*ml./min.*	*ml./min.*	*mEq./L.*	*mOsm./Kg. H_2O*
R.R.†	1.35	B	3.02	37	112	129	389	1.38	1.64	141	281
		NPB	5.63	44	248	101	569	2.04	3.59	141	279
		p-NPB	1.77	98	173	240	425	1.52	0.25	142	279
R.P.	1.75	B	1.78	127	226	686	1,220	4.02	−2.24	144	306
		NPB	1.27	126	160	644	819	2.68	−1.41	145	306
		p-NPB	0.80	167	134	777	622	2.03	−1.23	148	306
V.H.	1.60	B	0.80	100	80	639	508	1.77	−0.97	142	289
		NPB	2.20	55	121	330	728	2.48	−0.28	143	293
		p-NPB	1.23	94	116	485	596	2.04	−0.81	145	293
C.S.	1.62	B	0.44	112	50	686	304	1.05	−0.60	139	291
		NPB	1.28	109	139	540	691	2.41	−1.13	137	287
		p-NPB	0.93	140	130	638	595	2.09	−1.15	134	285
E.M.	1.55	B	1.66	168	278	670	1,110	4.05	−2.39	134	274
		NPB	4.03	68	274	250	1,010	3.64	0.39	134	277
		p-NPB	2.13	106	226	381	811	2.95	−0.82	134	275
V.D.	1.50	B	1.52	97	147	465	706	2.52	−1.00	130	280
		NPB	2.37	97	230	420	996	3.50	−1.13	132	285
		p-NPB	1.60	121	194	540	865	3.03	−1.43	134	285
I.D.	1.75	B	0.64	155	100	788	507	1.84	−1.20	130	276
		NPB	1.59	161	256	700	1,110	4.04	−2.45	131	276
		p-NPB	0.95	185	175	720	681	2.47	−1.52	130	276
E.B.	1.41	B	2.09	78	162	300	628	2.16	−0.07	142	291
		NPB	4.23	48	203	210	888	3.05	1.18	142	291
		p-NPB	1.35	167	226	520	704	2.42	−1.07	142	291
M.P.	1.50	B	0.98	308	301	838	817	2.84	−1.86	140	288
		NPB	1.09	320	349	832	908	3.14	−2.05	138	289
		p-NPB	0.23	337	78	864	201	0.69	−0.46	136	291
B.H.	1.42	B	2.29	69	158	284	651	2.28	0.01	141	285
		NPB	2.10	66	139	294	617	2.14	−0.04	141	288
		p-NPB	0.55	262	145	888	491	1.69	−1.14	141	290

* B = average of three basal periods; NPB = negative pressure breathing; p-NPB = post negative pressure breathing.
† Surreptitious water ingestion prior to the test could not be excluded.

TABLE II

*Hypertensive hydropenic patients: Response to negative pressure breathing**

Subject	Body surface area	Procedure	V	U_{Na}	$U_{Na}V$	U_{osm}	$U_{osm}V$	C_{osm}	C_{H_2O}	P_{Na}	P_{osm}
	M.²		*ml./min.*	*mEq./L.*	*μEq./min.*	*mOsm./Kg. H_2O*	*μOsm./min.*	*ml./min.*	*ml./min.*	*mEq./L.*	*mOsm./Kg. H_2O*
S.K.	1.69	B	0.66	155	102	716	472	1.64	−0.98	140	288
		NPB	0.73	148	107	700	509	1.78	−1.05	140	286
		p-NPB	0.80	149	119	688	550	1.91	−1.11	140	288
M.S.	1.35	B	1.42	175	248	606	862	3.08	−1.66	142	280
		NPB	6.60	44	290	130	858	3.06	3.54	140	281
		p-NPB	2.87	86	247	355	1,020	3.62	−0.75	143	281
H.W.	1.89	B	3.71	150	558	401	1,487	5.32	−1.61	142	280
		NPB	7.47	106	792	256	1,910	6.93	0.54	142	276
		p-NPB	4.13	168	694	382	1,570	5.75	−1.62	142	274
W.K.	1.92	B	0.86	254	218	750	643	2.25	−1.39	142	285
		NPB	1.40	132	185	408	572	2.05	−0.65	142	279
		p-NPB	0.63	286	180	798	501	1.78	−1.15	143	281
W.R.	1.91	B	0.74	136	100	650	481	1.65	−1.00	140	291
		NPB	0.75	117	90	633	478	1.64	−0.89	142	291
		p-NPB	0.61	118	72	642	394	1.35	−0.74	143	291
D.W.	1.78	B	1.75	122	213	590	1,932	3.44	−1.69	137	300
		NPB	4.60	105	483	405	1,790	5.97	−1.37	137	300
		p-NPB	1.80	108	194	444	800	2.67	−0.87	137	300
S.T.	1.55	B	0.52	184	96	700	364	1.35	−0.83	142	270
		NPB	1.16	197	228	742	860	3.15	−1.99	142	273
		p-NPB	1.00	123	123	600	600	2.20	−1.20	142	273
E.C.	1.66	B	1.22	53	65	360	439	1.51	−0.29	142	291
		NPB	3.50	69	242	249	871	3.00	0.50	142	290
		p-NPB	0.76	64	49	396	301	1.04	−0.28	142	288
D.M.	1.67	B	0.56	212	119	875	490	1.76	−1.20	142	278
		NPB	0.88	180	158	784	689	2.49	−1.61	144	277
		p-NPB	0.71	205	145	856	606	2.18	−1.47	143	278
E.B.	1.70	B	1.22	50	61	468	571	1.92	−0.70	144	297
		NPB	1.15	54	62	490	564	1.90	−0.75	144	297
		p-NPB	0.49	31	15	594	292	0.99	−0.49	144	297

* B = average of three basal periods; NPB = negative pressure breathing; p-NPB = post negative pressure breathing.

the probability (p) values of significance, as determined by the simpler nonparametric test, have in all instances been associated with analogous values of significance as obtained by the "t" test of parametric analysis.

RESULTS AND DISCUSSION

In opening this discussion it must be re-emphasized, first, that where others have examined the effects of NPB in moderately (and variably) hydrated subjects,[1] we have endeavored to bring our subjects into a fairly steady hydropenic state, for the reason that we were interested less in evoking a substantial water diuresis than in examining the effects of NPB on natriuresis in hypertensive and prehydrated subjects; the former are known to give exaggerated natriuresis to water (12), while the natriuretic response of prehydrated subjects appears to be inhibited by rehydration (13). From the studies of others and from our own results, it is clear that NPB has at best a very mild diuretic effect, and in retrospect it is perhaps surprising that we obtained as distinct a diuretic effect in hydropenia as we did.

Second, under the oliguric conditions of our observations, all constituents of the urine are present in a relatively concentrated state and any abrupt increase in urine flow, consequent to an increased excretion of free water, will lead to a substantial dead space error as concentrated urine, formed at a pre-existing low flow, is pushed out of the collecting ducts, pelvis and ureters at an accelerated rate.

Third, an increase in urine flow at oliguric levels will be accompanied by a substantial increase in excretion of urea and hence in $U_{osm}V$, a result which is incidental rather than specifically related

[1] Sieker, Gauer and Henry (1) gave a priming dose of 300 ml. of 0.14 per cent NaCl and 50 to 100 ml. every half hour; Surtshin, Hoeltzenbein and White (2) gave 300 ml. of 0.14 per cent NaCl; Boylan and Antkowiak (3) gave 50 to 150 ml. of water every half hour; and Murdaugh, Sieker and Manfredi (4) gave one glass of water every two hours for six hours prior to the study.

to NPB. However, we have not followed urea excretion in these studies.[2]

Lastly, in hydropenia the urine is of course hyperosmotic to the blood; in previous discussions (18–20), the water abstracted in attaining this hyperosmotic state has been designated as $T^c_{H_2O}$, and calculated as

$$T^c_{H_2O} = C_{osm} - V.$$

However, except for sign, a decrease in $T^c_{H_2O}$ is mathematically (and physiologically) indistinguishable from an increase in free water clearance, calculated as

$$C_{H_2O} = V - C_{osm}.$$

Since an obvious inconvenience attaches to a recurring change in designation from $T^c_{H_2O}$ to C_{H_2O} as the urine passes from the hyperosmotic to the hypo-osmotic state (and vice versa), we have adhered to the latter equation and simply recorded negative and positive values for C_{H_2O}. Whatever semantic difficulties may be raised by talking about a "negative renal clearance," terminology seems to be evolving in this direction in the writings of others, and the advantages of a uniform method of expression in our immediate study are obvious.

[2] An increase in $U_{osm}V$ (and consequently C_{osm}) due to "washout" of urea solely as a result of increased urine flow would tend to mask a portion of the actual free water excretion that occurred in response to NPB. Therefore, actual values for free water clearance (C_{H_2O}) are possibly higher than those calculated.

Control subjects

Table I gives our observations on 10 control normotensive subjects. The basal values repre-

TABLE III

*Normal prehydrated subjects: Response to negative pressure breathing**

Subject	Body surface area	Procedure	V	U_{Na}	$U_{Na}V$	U_{osm}	$U_{osm}V$	C_{osm}	C_{H_2O}	P_{Na}	P_{osm}
	M.²		*ml./min.*	*mEq./L.*	*μEq./min.*	*mOsm./Kg. H_2O*	*μOsm./min.*	*ml./min.*	*ml./min.*	*mEq./L.*	*mOsm./Kg. H_2O*
P.R.	1.80	B	2.26	45	101	300	678	2.30	−0.04	138	294
		NPB	5.47	49	268	222	1,210	4.09	1.38	139	297
		p-NPB	0.64	176	112	872	557	1.86	−1.22	140	300
R.S.	1.75	B	1.13	68	76	391	442	1.58	−0.45	139	279
		NPB	2.83	40	113	162	458	1.63	1.20	139	281
		p-NPB	0.73	183	134	721	529	1.88	−1.15	139	282
L.B.	1.45	B	1.35	146	197	582	786	2.88	−1.53	143	273
		NPB	8.93	32	286	102	910	3.34	5.59	143	273
		p-NPB	1.20	220	264	707	849	3.10	−1.90	143	274
K.P.	2.08	B	2.99	59	177	286	856	3.04	−0.05	131	282
		NPB	6.60	42	277	172	1,130	4.03	2.57	131	282
		p-NPB	0.84	218	183	1,048	880	3.12	−2.28	131	282
J.B.	1.35	B	0.62	138	86	591	368	1.32	−0.70	140	279
		NPB	2.47	70	173	214	528	1.87	0.60	141	283
		p-NPB	0.47	225	105	707	330	1.16	−0.69	142	285
E.D.	1.75	B	2.43	76	185	304	740	2.66	−0.23	142	278
		NPB	4.20	52	218	202	849	3.04	1.16	142	279
		p-NPB	0.93	140	131	576	537	1.92	−0.99	142	279
L.J.	1.70	B	1.24	69	86	538	667	2.34	−1.10	141	285
		NPB	1.80	63	113	388	699	2.45	−0.65	139	285
		p-NPB	0.77	145	111	780	598	2.10	−1.33	140	285
A.B.	1.73	B	1.07	208	223	751	804	2.79	−1.72	143	288
		NPB	1.49	200	298	678	1,010	3.47	−1.98	143	291
		p-NPB	0.96	290	278	952	914	3.12	−2.16	143	293
G.D.	1.57	B	2.23	56	124	205	458	1.61	0.62	142	285
		NPB	3.95	45	182	176	706	2.45	1.50	140	288
		p-NPB	0.60	158	95	595	357	1.20	−0.60	138	297
S.B.	1.62	B	1.01	86	87	469	474	1.69	−0.68	145	281
		NPB	2.53	86	218	309	782	2.72	−0.19	142	288
		p-NPB	1.20	110	132	388	466	1.62	−0.42	142	288
Avg. basal values:											
Controls			1.52		161		684	2.39	−0.87	138	286
Hypertensives			1.26		178		684	2.39	−1.13	141	286
Prehydrated			1.63		134		627	2.22	−0.60	140	282

* B = average of three basal periods; NPB = negative pressure breathing; p-NPB = post negative pressure breathing.

sent the averages of three 15 minute urine collections prior to NPB. That a fairly steady rate of urine flow had been attained by our protocol is shown by the fact that V for all 30 basal periods averaged 1.52 ml. per minute, while the 10 periods immediately preceding NPB averaged 1.47 ml. per minute. Eight subjects responded to NPB by an increase in V. In three subjects (R.R., E.M. and E.B.) C_{H_2O} was positive and in one subject, (V.H.), the change in C_{H_2O} was directionally positive. The change to a more negative C_{H_2O} in Subjects C.S. and I.D. may reflect a falsely high C_{osm}, the consequence of "washout" of urea, which conceivably could obscure a directionally positive C_{H_2O}. The difference between the average values of C_{H_2O} for all 10 subjects, as between the basal and NPB periods (− 0.87 and − 0.33 ml. per minute, respectively), was not statistically significant, but this is not surprising since many uncontrollable factors can influence water diuresis of small magnitude, such as differences in psychic reaction, duration and degree of hydropenia and so forth. Moreover, it is possible that some persons are insensitive to NPB—Boylan and Antkowiak (3) report failure to obtain water diuresis in four out of 16 moderately hydrated subjects. Consequently the fact that the mean value of C_{H_2O} did not increase significantly in our hydropenic subjects is less important than the qualitative fact that four out of our 10 subjects gave an absolute or directionally positive change in C_{H_2O}.[3]

With respect to sodium excretion, the basal values of $U_{Na}V$ varied widely (50 to 301 μEq. per minute), which is not unexpected in view of the well known variability of this term. Nevertheless our protocol achieved a steady state in each subject, as indicated by an average value of $U_{Na}V$ for all 30 basal periods of 161 μEq. per minute, as compared with 160 μEq. per minute for the 10 periods immediately preceding NPB. More significance can therefore be attached to changes in $U_{Na}V$ associated with NPB than to the absolute values of this term.

When an increase in $U_{Na}V$ was coincident with an increase in C_{H_2O} (R.R., E.B. and V.H.), the former undoubtedly reflects dead space error. In three subjects (C.S., V.D. and I.D.), $U_{Na}V$ increased in the absence of an increase in C_{H_2O}; since the fraction of U_{osm} supplied by U_{Na} remained nearly constant, however, the increase in $U_{Na}V$ (and $U_{osm}V$) may be attributable to a slight increase in filtration rate (4).

Taking all observations, the changes in $U_{Na}V$ ranged from − 66 (R.P.) to + 156 (I.D.) μEq. per minute, and algebraically averaged + 51 μEq. per minute. This figure is not significant statistically ($p > 0.05$) and we conclude that NPB is without effect on sodium excretion in hydropenic normotensive subjects. This conclusion is in agreement with the studies of Sieker, Gauer and Henry (1), Surtshin, Hoeltzenbein and White (2), Boylan and Antkowiak (3) and Murdaugh, Sieker and Manfredi (4).[4]

Hypertensive subjects

Table II gives our observations on 10 hypertensive subjects. (The data are treated in the same manner as are the control subjects.) Again a steady state in respect to V and $U_{Na}V$ in the basal periods was achieved by our protocol, as shown by averages of 1.26 ml. per minute and 178 μEq. per minute in the 30 basal periods prior to NPB, as compared with 1.28 ml. per minute and 177 μEq. per minute in the 10 periods immediately preceding NPB.

Eight subjects responded to NPB by a substantial increase in V; only in three (M.S., H.W. and E.C.), however, did C_{H_2O} become positive, though the directional change was positive in three others (W.K., W.R. and D.W.). The average value of C_{H_2O} did not increase significantly (basal, − 1.13; NPB, − 0.37 ml. per minute), but as

[3] The average change in V (+ 1.06 ml. per minute) associated with NPB is statistically significant ($p < 0.05$); but the changes in the component terms, C_{osm} (+ 0.52 ml. per minute) and C_{H_2O} (+ 0.54 ml. per minute), are not individually significant ($p > 0.05$ in both instances). This illustrates one of the ambiguities of statistical analysis: two variables, neither of which alone varies by a significant amount, can, when added apparently in a random manner, yield a sum which in the average does vary by a significant amount. Hence the futility of trying to prove, by statistical analysis alone, that a compound variable differs, as between two experimental conditions, by a physiologically significant amount. The converse argument is, of course, not true: if the difference is physiologically significant, it should also be statistically significant by one or more of the conventional criteria.

[4] Plasma osmolality (P_{osm}) did not change during NPB in any of our subjects, confirming Boylan and Antkowiak (3).

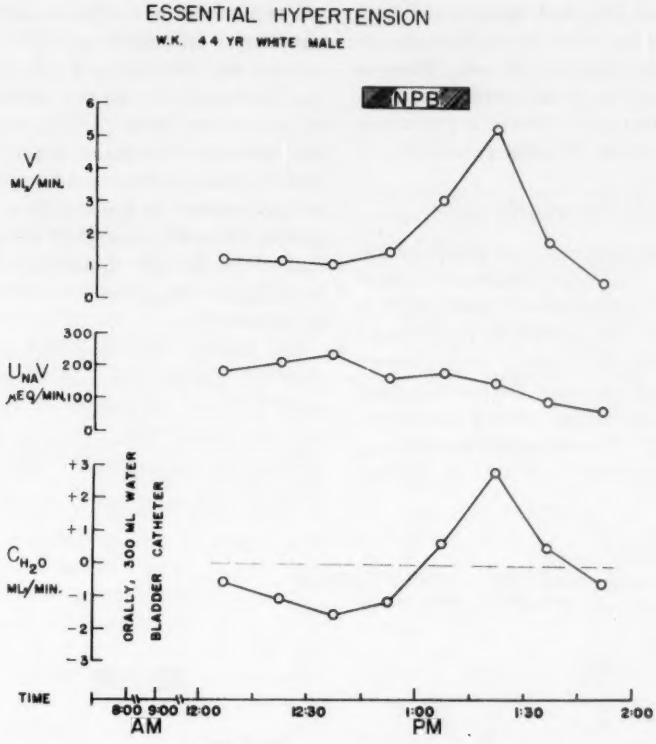

FIG. 1. NPB FOLLOWING MODERATE HYDRATION

noted above, in view of the hydropenic nature of our subjects this is less important than the qualitative fact that six out of 10 subjects gave an absolute or directionally positive change in this function. The diuretic effect of NPB appears to be as substantial in hydropenic hypertensive subjects as in normotensive subjects.

Basal sodium excretion again varied widely, from 61 to 558 μEq. per minute. Maximal changes related to NPB ranged from − 33 (W.K.) to + 270 μEq. per minute (D.W.) and algebraically averaged + 86 μEq. per minute, again a figure which is not statistically significant. We conclude that NPB has no natriuretic effect in hydropenic hypertensive subjects not explicable by dead space error.

Statistically, urine flow ($V = + 1.56$ ml. per minute) was the only function in which the change was significant ($p < 0.05$), but again the component average terms, C_{osm} (+ 0.80 ml. per minute) and C_{H_2O} (+ 0.76 ml. per minute) did not separately show a significant increase ($p > 0.05$).

The importance of the state of hydration on the response to NPB is suggested by Figure 1, which shows a second test on W.K. who, as reported in Table II, gave only a barely significant response (− 1.39 to − 0.65 ml. C_{H_2O} per minute) in the hydropenic state. When tested four days later after moderate hydration (300 ml. water followed by 50 ml. every half hour), C_{H_2O} increased from − 1.37 to + 2.75 ml. per minute. This response also indicates (as do our other data) that hypertensive subjects may be expected to respond to NPB as do normotensive subjects.

Contrariwise, the hazard of attributing natriuresis in a hypertensive subject to a particular stimulus is illustrated by Figure 2. D.B., a 56 year old white, female patient, was instructed to forego fluids from the night before the test. She came to the Research Unit at 8:00 a.m. and at

9:30 a.m. the urine flow was suspiciously high (2.33 ml. per minute). After interrogation she admitted having drunk coffee at 6:30 a.m. Whether attributable to the coffee, to catheterization, or to interrogation, she thereafter showed a natriuresis which at its peak reached 802 μEq. per minute.

Prehydrated normotensive subjects

In contrast to the previous two groups, NPB induced a positive free water clearance in seven out of 10 prehydrated subjects (Table III); in these seven subjects, the increase in C_{H_2O} ranged from 1.30 to 7.12 ml. per minute. In two others (L.J. and S.B.), C_{H_2O} showed a directionally positive change, while one subject (A.B.) showed increased antidiuresis. The mean increase in C_{H_2O} for the nine subjects was 2.06 ml. per minute ($p < 0.01$).

The increase in sodium excretion in these 10 prehydrated subjects ranged from 27 to 167 μEq. per minute and averaged 80 μEq. per minute; C_{osm} increased through the range of 0.05 to 1.79 ml. per minute (average, 0.70 ml. per minute), in part because of increased $U_{Na}V$ and possibly in part because of increased urea excretion. The average increase in V was 2.41 ml. per minute, reflecting increased C_{H_2O} (1.71 ml. per minute) and C_{osm} (0.70 ml. per minute). All three terms ($+\Delta C_{H_2O}$, $+\Delta C_{osm}$ and $+\Delta V$) are statistically significant.

The average basal value of $U_{Na}V$ in these subjects was 134 μEq. per minute, the average peak value, 215 μEq. per minute, figures which, excluding bicarbonate, represent only some 1 and 1.6 per cent, respectively, of the estimated filtered load of NaCl (102 mEq. per L. $\times$ 125 ml. per minute

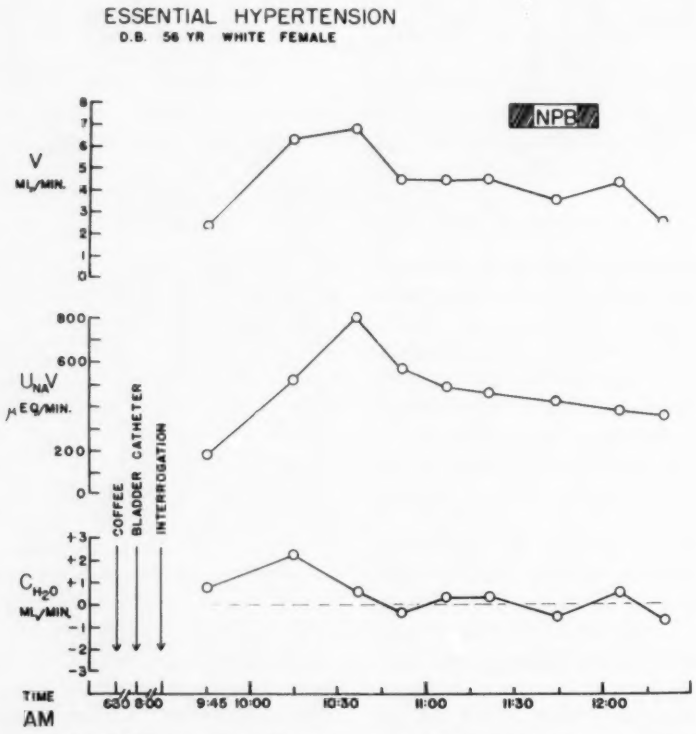

FIG. 2. SPONTANEOUS NATRIURESIS IN A HYPERTENSIVE PATIENT

The relative large urine flow at 9:30 a.m. suggested moderate hydration. The subject admitted to prior ingestion of coffee.

or 12.7 mEq. per minute). We believe that an increase in $U_{Na}V$ of this magnitude is most likely the unavoidable consequence of dead space error related to the increase in C_{H_2O}. (The transience of the diuretic action of NPB precludes studies of $U_{Na}V$ at a constant rate of urine flow.) For this reason we conclude that, in contrast to the effect of prehydration in facilitating the water diuresis produced by NPB, prehydration does not predispose the subject to natriuresis under this stimulus.

Thus the diuretic and natriuretic phenomena discussed elsewhere (21) may be dissociated with respect to excitatory stimuli. The diuretic effect of NPB is demonstrable in hydropenic hypertensive subjects and it is exaggerated (or facilitated) in prehydrated normotensive subjects who are also in the hydropenic state, but in neither group does NPB induce a natriuretic response. The afferent pathways from the Henry-Gauer reflex may be envisioned as communicating with the neurohypophyseal centers which mediate water diuresis, but apparently they do not communicate with neural centers having to do with natriuresis.

Subordinately, it is of interest to note that our basal values for control, hypertensive, and prehydrated subjects are very similar, as is shown at the bottom of Table III.

SUMMARY AND CONCLUSIONS

1. The effect of negative pressure breathing (NPB) on sodium, total solute and water excretion was observed in hydropenic normotensive subjects, hydropenic hypertensive subjects and normotensive subjects who had been prehydrated by water loading eight to nine hours before NPB.

2. Normotensive and hypertensive subjects responded to NPB with increased urine flow primarily because of increased free water clearance (C_{H_2O}). The increments observed in osmolal clearance (C_{osm}) may be attributable to "washout" of urea in consequence of increased urine flow. Significant increases in sodium excretion did not occur.

3. NPB in prehydrated subjects induced a substantial increase in free water clearance, though at the time of NPB the subjects were in an antidiuretic state comparable to hydropenic nonprehydrated subjects. The small increase in sodium excretion can be explained by dead space error.

4. It is concluded that, under the conditions of this study, NPB is not a stimulus that leads to excessive natriuresis in subjects (hypertensive and prehydrated) otherwise predisposed to natriuresis.

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IDENTIFICATION OF METABOLITES OF SULFOBROMOPHTHALEIN *

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Sulfobromophthalein (BSP) is normally cleared from the blood by the liver and excreted into the bile. Brauer, Krebs and Pessotti (1) originally reported that part of the pigment appearing in dog bile was chromatographically different from the injected dye. Our studies using different chromatographic techniques indicate that in man the bulk of the recovered pigments (65 to 75 per cent, according to estimates based on their absorption at 575 mμ) consists of two metabolites. Similar metabolites are normally found in the urine and trace amounts also appear in the serum. The absorption spectra in the visible range and the indicator characteristics of BSP and the metabolic products were indistinguishable (2).

The liver apparently is capable of producing the metabolites, since they have been demonstrated in bile from preparations of isolated rat liver perfused with BSP (3, 4). Variations in the concentration of BSP metabolites in serum and urine of patients with liver disease are reported elsewhere (5).

Since the chemical difference between the free dye and its metabolites elucidates in part the mechanism of hepatic function involved in BSP clearance, characterization of this structural difference has received considerable attention. BSP is a derivative of phenolphthalein, therefore both compounds could be excreted by the same pathway, that is, by conjugation with glucuronic acid (6). This mechanism for BSP clearance appears unlikely since the metabolites were stable to hydrolysis with β-glucuronidase (4, 7), with dilute acid and with dilute base, and gave a negative Dische reaction for uronic acid (4). Furthermore, both of the major metabolites in human bile were associated with ninhydrin-positive substances, whose identification was complicated by the presence of high concentrations of ninhydrin-positive impurities arising from the bile itself (3). Several recent reports have also indicated that the BSP metabolites contain a ninhydrin-positive moiety (8–10). The present study reports the presence of glycine and glutamic acid as possible contaminants of the BSP metabolites and indicates that BSP is excreted as a mercaptide with cysteine or the peptide glutathione.

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METHODS AND MATERIALS

Experimental. Human subjects with draining bile fistulas were injected with 5 mg. of BSP per Kg. of body weight. Control specimens of bile were taken immediately before the dye was injected. Test specimens, in equal volume, were collected during a 30 to 60 minute interval after the injection.

Isolation of the crude metabolites. In initial experiments, the metabolites were isolated as follows. The total BSP pigments were extracted from bile with three volumes of acetone. The acetone extract was taken to dryness *in vacuo*, then dissolved in water (approximately 0.2 ml. per ml. of initial bile). The solution was chromatographed on Whatman 3 MM paper in an ascending system employing *tert*-butanol-water (1.73:1 v/v) for six to 15 hours. The BSP bands were developed with ammonia fumes (or if permanent records were desired, by dipping the paper in a fresh saturated solution of sodium hydroxide in 90 per cent ethanol). After the position of the metabolites had been determined with ammonia, the bands were cut out and individually eluted with water. The resultant solutions were then taken to dryness.

Purification of the metabolites. Purified pigments were prepared by chromatography in three systems. Activated alumina (Alcoa, 80 to 200 mesh) was washed 10 times in two volumes of water and once in acetone. During each washing period, the larger particles were allowed to settle for one minute; the supernatant was then decanted and discarded. The dried alumina was suspended in a loading solution consisting of two volumes of acetone to one volume of water. Columns, 3.4 cm. (outer diameter) by 6 cm., were prepared by gravity packing. The dried acetone extract from 60 ml. of bile was sus-

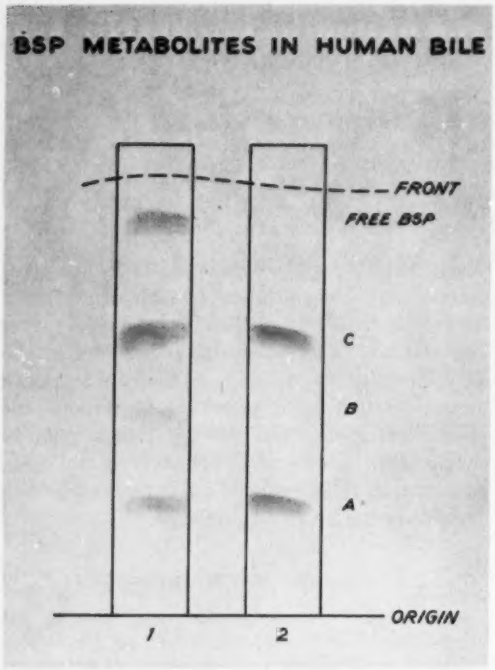

FIG. 1. PAPER CHROMATOGRAPH OF BSP PIGMENTS IN HUMAN BILE (DRAWING)

Ascending system employing *tert*-butanol-water was used. *1.* BSP color developed with NaOH; *2.* ninhydrin color developed with 0.1 per cent ninhydrin in ethanol.

pended in 45 ml. of loading solution and passed through the column. The column was then washed with an additional 200 ml. of the pure loading solution. Unconjugated BSP, amino acids and bile pigments were eluted with 600 ml. of a solution of concentrated ammonium hydroxide, water and acetone (4:46:50 v/v). The conjugated BSP remaining on the column was eluted with 125 ml. of acetone-water (1:9 v/v). The eluate was taken to dryness, suspended in a minimal amount of water and subjected to ascending paper chromatography on Whatman 3 MM paper [1] in a solvent system consisting of *n*-butanol-water (1.48:1 v/v) made to a single phase solution by the addition of 0.27 volume of acetic acid. This system further removed traces of ninhydrin-positive impurities. After elution with water, the metabolites were separated by chromatography in the *tert*-butanol-water system used originally. The individual metabolites were then eluted with water, taken to dryness and stored *in vacuo*.

The equal volume of control bile obtained from each subject immediately before injection of BSP was treated similarly. Fractions corresponding in mobilities to the individual metabolites were isolated by employing the three chromatographic systems just described. The chemical characteristics of the control fractions were compared with those of the pigment fractions obtained from the bile containing BSP.

Hydrolysis of the metabolites. In initial experiments the individual metabolites were hydrolyzed in 6 N hydrochloric acid in a sealed glass tube at 120° C. for 18 hours. After being repeatedly dried and redissolved in water to drive off traces of remaining hydrochloric acid, the hydrolysate was subjected to chromatography in the *tert*-butanol-water solvent system. The individual amino acids were subsequently identified by two-dimensional chromatography (11). Since hydrolysis with hydrochloric acid destroys several amino acids, including cysteine (12), this method was subsequently discarded in favor of hydrolysis with 48 per cent hydrobromic acid in a sealed tube at 120° C. for 18 hours.

Hydrogenolysis with Raney-nickel. Raney-nickel catalyst W-2 was prepared as described by Mozingo (13). A sample of metabolite containing approximately 0.4 mg. of BSP (by weight) was dissolved in 0.2 ml. of water and mixed with 200 mg. of Raney-nickel suspended in 2.5 ml. of ethanol. After being refluxed for two hours, the mixture was centrifuged and the supernatant was removed. The precipitate was washed two times with duplicate washes of 0.5 ml. of 50 per cent ethanol and five times with 0.5 ml. of 10 per cent ammonium hydroxide. The supernatant and washes were combined and taken to dryness *in vacuo*. "Desulfurated glutathione" was prepared by substituting glutathione for the sample of the metabolite in the above procedure.

Synthesis of BSP-amino acid complexes. A solution consisting of 0.1 mMole of free BSP and 0.1 mMole of cysteine, adjusted to 2 ml. with water, was heated in a sealed tube at 120° C. for 18 hours. The reaction mixture was taken to dryness and free BSP and the unreacted amino acids were removed by the same three chromatographic systems used for the BSP metabolites. The complexes, if any, were eluted with water and taken to dryness *in vacuo*. Complex formation of BSP and the following amino acids or peptides was similarly attempted: cystine, glutathione, tryptophan, tyrosine, phenylalanine, glycine, glutamic acid, valine, alanine, serine, threonine, methionine, lysine hydrochloride and leucine.

[1] To reduce ninhydrin-positive impurities, the paper was first washed 10 times with glass-distilled water.

RESULTS

The BSP metabolites obtained from bile reacted positively with ninhydrin (Figure 1). The possibility that the conjugates were complexes with protein is unlikely, since most of the proteins were either precipitated during the initial acetone extraction of bile or held at the origin in the chromatographic systems employed.

When BSP-A and BSP-C were eluted from the initial paper chromatograms and hydrolyzed in hydrochloric acid, ninhydrin-positive substances

TABLE I

Release of ninhydrin-positive material from BSP metabolites after hydrolysis with HCl

	Before HCl			After HCl		
	Control	Metabolite	Metabolite minus control	Control	Metabolite	Metabolite minus control
	μg. N/ml. of fraction*			*μg. N*/ml. of fraction*		
BSP-A	1.1	8.1	7.0	19.9	30.2	10.3
BSP-A	3.0	15.0	12.0	17.2	31.0	16.0
BSP-A	2.4	12.6	10.2	12.6	25.7	13.1
BSP-C	2.0	7.1	5.1	10.2	15.3	5.1

* Quantitative ninhydrin determined by method of Moore and Stein (14). Glycine was used as the standard for calculating μg. N.

were released, which when analyzed by two-dimensional chromatography proved to be primarily glycine and glutamic acid. However, when samples of normal bile (which did not contain BSP) were chromatographed and fractions corresponding in mobility to the metabolites were eluted and hydrolyzed, large quantities of glycine and glutamic acid were again found. The amounts of ninhydrin-positive material released from the crude BSP fractions and from identical fractions of control bile before and after hydrolysis with hydrochloric acid are compared in Table I. (No attempt was made to establish the ratio of BSP to amino nitrogen in this crude mixture.) The content of ninhydrin-positive material in the BSP metabolites, which was higher than that of the control fractions before hydrolysis, was increased two- to fivefold after hydrolysis. Analysis of the controls, however, showed that most of this amino nitrogen came from the bile or paper (as glycine and glutamic acid) rather than from the BSP metabolites. The metabolites required further purification before their ninhydrin-positive constituents could be identified.

The chromatographic paper used in most of our studies contained considerable amounts of glycine and glutamic acid which were demonstrable only after acid hydrolysis. These impurities were substantially reduced by the described washing procedure. The electrophoretic mobility of bile-acetone extracts containing BSP were extensively studied with three systems using 0.25 M acetic acid as the electrolyte: Durrum paper electrophoresis (15), hanging curtain electrophoresis (16) and starch block electrophoresis (17). The patterns obtained with the three systems were essentially the same. Unconjugated BSP moved rapidly toward the anode; the ninhydrin-positive metabolites moved less rapidly toward the anode and were usually indistinguishable from each other; large quantities of ninhydrin-positive impurities remained at the origin or moved toward the cathode. For large-scale preparations, electrophoresis was eventually abandoned in favor of chromatography on alumina columns, followed by chromatography in the two systems, butanol-water and *n*-butanol-water-acetic acid. On three occasions, the molar ratio of BSP to ninhydrin-positive amino nitrogen (with cysteine as a standard) of each metabolite ranged from 0.8 to 1.2. Control bile purified by this procedure was ninhydrin-negative *before* hydrolysis. Figure 2A shows the results obtained after hydrolysis of the purified metabolites with hydrochloric acid. Glycine and glutamic acid were still found in the controls, as well as in the fractions containing the metabolites. When the concentrations of purified pigment were greater than 0.25 mg. per square cm., a faint ninhydrin-positive streak corresponding to cysteine was usually detectable in the BSP fractions but not in the controls. The often reported (12) destruction of cysteine by hydrochloric acid was demonstrated when glutathione, a peptide containing glutamic acid, glycine and cysteine in equal quantities, was hydrolyzed in hydrochloric acid and chromatographed (Figure 2A). At low concentrations so much of the cysteine was destroyed that only glycine and glutamic acid were detectable.

Hydrolysis with hydrobromic acid

Some amino acids are comparatively stable during hydrolysis with hydrobromic acid (18). Furthermore, this reagent can be more effective than

hydrochloric acid in hydrolyzing mercaptides (19). In the chromatographic patterns obtained after hydrobromic acid hydrolysis, cysteine was detectable as a discrete well-defined spot arising from both BSP purified metabolites (Figure 2B). The presence of cysteine was the only qualitative difference between the metabolites and the controls.

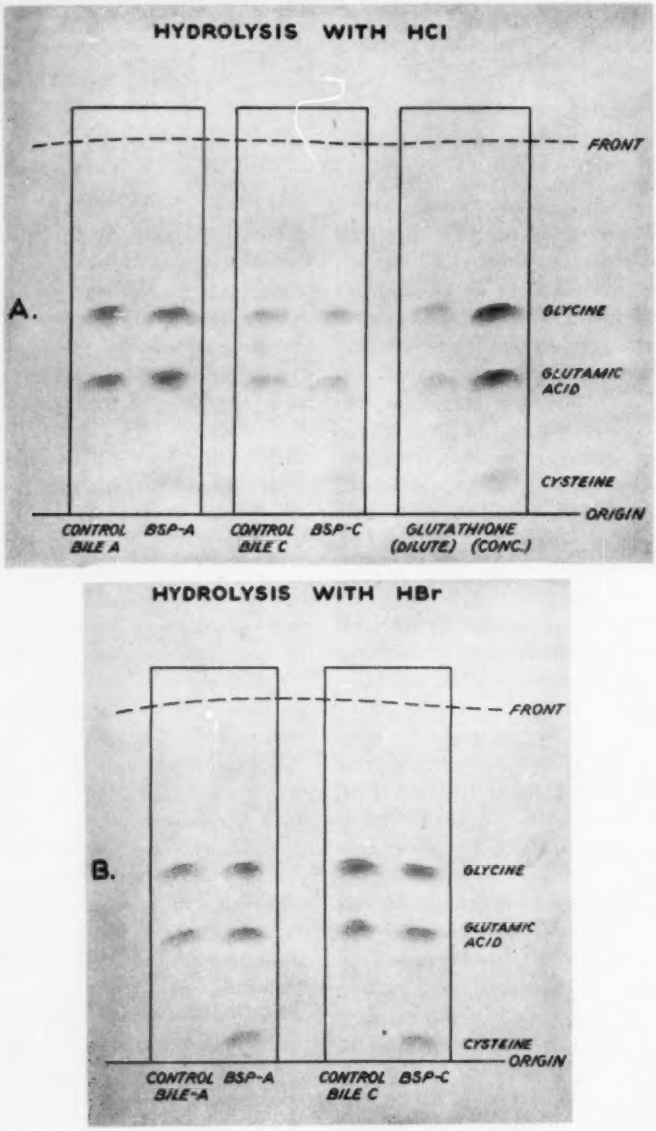

FIG. 2. CHROMATOGRAPHY OF NINHYDRIN-POSITIVE SUBSTANCES ARISING FROM BILE AND BSP METABOLITES

Ascending system employing *tert*-butanol-water was used. A. Hydrolysis in 6 N HCl at 120° C.; B. hydrolysis with 48 per cent HBr at 120° C.; C. hydrogenolysis with Raney-nickel.

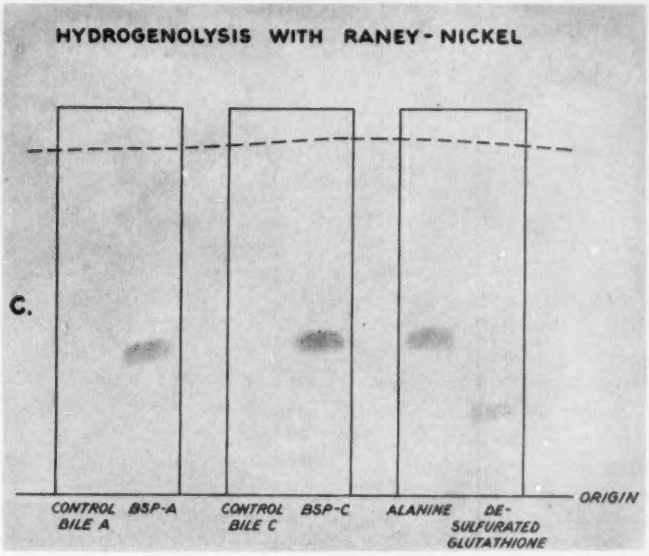

FIG. 2.—*Continued*

Raney-nickel hydrogenolysis

Hydrogenolysis with Raney-nickel breaks the carbon-sulfur bond in mercaptide linkages (20). If the BSP metabolites are conjugated with cysteine through the cysteine-sulfur bond, Raney-nickel treatment should result in the degradation of the cysteine and the release of desulfurated cysteine (alanine). Furthermore, since this treatment does not hydrolyze peptide bonds, the interference of amino acids arising in the controls after hydrolysis with hydrochloric acid or hydrobromic acid is eliminated. When either purified BSP-A or BSP-C was treated with Raney-nickel and the resultant product was chromatographed (Figure 2C), only one major ninhydrin-positive spot was detected. This material was chromatographically identical to alanine after two-dimensional chromatography. There was no spot corresponding to desulfurated glutathione (the *assumed* product of BSP-glutathione degradation by Raney-nickel). No significant amount of ninhydrin-positive material was demonstrable in the controls after treatment with Raney-nickel.

Staining with dichromate-silver nitrate

Knight and Young reported that mercaptides and compounds with an available sulfhydryl group, when stained with potassium dichromate-silver nitrate, give a positive reaction characterized by an orange spot on a dark brown background (21). Both BSP-A and BSP-C reacted positively, while similar fractions from control bile were negative.

Synthesis of BSP-amino acid complexes

When BSP was incubated at 120° C. with 14 different amino acids, only cysteine (or cystine) produced a complex with the pigment.[2] After elimination of unreacted BSP and cysteine on the alumina column, the product contained BSP and cysteine in a molar ratio of 1 to 1. When chromatographed in *tert*-butanol-water, the BSP-cysteine complex moved as two spots, corresponding in mobility to purified BSP-A and BSP-C, respectively. Both spots were ninhydrin-positive, stained with dichromate-silver nitrate and produced alanine (identified by two-dimensional chromatography) after treatment with Raney-nickel. When the synthetic mixture was added to bile, the chromatographic pattern was identical to that of the natural products (Figure 3). Pyrolysis of BSP with glutathione also produced ninhydrin-

[2] By this procedure phenolphthalein also formed complexes with cysteine. Presumably then, the cysteine may conjugate directly with the phenolphthalein core of the BSP molecule.

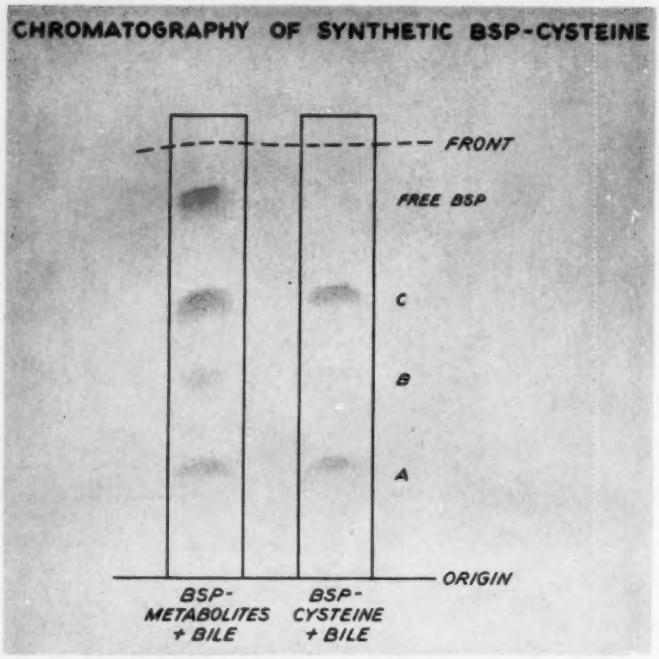

FIG. 3. CHROMATOGRAPHY OF BSP METABOLITES AND SYNTHETIC BSP-CYSTEINE COMPLEXES

positive synthetic complexes chromatographically similar to BSP-A and BSP-C.

DISCUSSION

Hydrolysis of the BSP metabolites appearing in human bile with hydrobromic acid, treatment with Raney-nickel, and staining with dichromate-silver nitrate strongly suggest that BSP is excreted, at least in part, as conjugates containing cysteine. Synthetic complexes of BSP and cysteine have the same chromatographic mobilities as the natural metabolites.

The fact that hydrolysis of the metabolites with hydrochloric acid destroys most of the cysteine may explain why the presence of this amino acid was not noted by others (8, 10) who used only hydrochloric acid hydrolysis. The appearance of glycine and glutamic acid after hydrolysis of the metabolites may be misleading under circumstances where control bile is not similarly treated (10). Hydrolysis of normal bile purified by our procedures demonstrated that much of the glycine and glutamic acid in the fractions containing the BSP metabolites may arise from the bile itself or from impurities in the chromatographic paper. However, conclusions based on comparisons of the quantities of amino acids appearing in purified normal bile and in bile containing BSP after acid hydrolysis are also equivocal. The control samples are necessarily taken about 30 minutes before the experimental samples and the two may not be identical in composition. For this reason, the persistent presence of glycine and glutamic acid cannot, at present, be ignored. If glycine and glutamic acid are involved in BSP metabolism, they may be either in combination with the cysteine as a peptide or exist as independent complexes with the same chromatographic mobility as BSP-cysteine mercaptide. In view of the numerous chromatographic systems employed in this study, the latter possibility is unlikely. Furthermore, a conjugation of the acidic BSP molecule with glycine would most likely take place through a peptide linkage, which would result in a ninhydrin-negative compound. The association of cysteine, glycine and glutamic acid with the metabolites

suggests that glutathione could be involved in BSP metabolism. Although it has never been satisfactorily demonstrated, glutathione has often been suggested as a precursor for cysteine conjugation (*22, 23*). Bray and Franklin showed that liver slices were capable of producing cysteine mercaptides from previously formed glutathione mercaptides (*23*). We observed that the synthetic BSP-glutathione complexes have chromatographic mobilities similar to those of the naturally occurring metabolites. Isotopic studies, currently under way, may clarify the role of glycine and glutamic acid in the metabolism of BSP.

BSP contains four bromine atoms on one of its three aromatic rings. Precedent for the conjugation of halogenated aromatic substances with cysteine is well known. Two sites for the attachment of cysteine to the BSP molecule are possible: a complex with the SH group of the cysteine molecule may occur *a*) on a free position of an aromatic ring or *b*) after substitution for one of the halogens (*24*). Krebs and Brauer reported that the ratio of bromine to S^{35} in BSP and in the metabolites is the same (*7*). If so, this observation indicates that no bromine has been lost and that the complexes are at the free positions of the ring. The demonstration that two major complexes can be synthesized from BSP and cysteine suggests two possibilities: that cysteine may complex to different available positions on the BSP molecule or that more than one molecule of cysteine may attach to each BSP molecule. The approximately 1:1 molar ratio of BSP to cysteine in both metabolites favors the former possibility.

The demonstration that BSP is conjugated with cysteine suggests that other tissues may be capable of metabolizing the dye. Mills and Wood (*25*), utilizing tissue slices, have shown that cysteine conjugation reactions can occur in kidney. Indication of the extrahepatic metabolism of BSP is further suggested by the fact that BSP metabolites account for 25 per cent of the dye excreted in the urine of hepatectomized dogs (*26*).

After the uptake of circulating BSP by the liver, a significant delay occurs before the pigment is secreted into the bile (*27*). Apparently at least two processes, both of which may require active enzyme systems, are involved in the clearance of BSP. It is not inconceivable that the conjugation of BSP with cysteine or cysteine-containing peptides may be the rate-determining step for either the uptake of the dye by the liver or its eventual secretion into the bile.

SUMMARY

The two major ninhydrin-positive metabolites of sulfobromophthalein appearing in human bile were isolated by column and paper chromatography. Their chemical characteristics were studied as follows:

1. Hydrolysis of the purified metabolites with concentrated hydrochloric acid at 120° C. revealed the presence of the amino acids, glycine, glutamic acid and possibly cysteine. Similar treatment of control bile containing no sulfobromophthalein also resulted in the appearance of considerable amounts of glycine and glutamic acid.

2. Hydrolysis of the metabolites with hydrobromic acid more clearly demonstrated the presence of cysteine, which was not detectable in the control bile after similar treatment. Both controls and metabolite fractions contained glycine and glutamic acid.

3. Treatment of both metabolites with Raney-nickel resulted in release of desulfurated cysteine (alanine). Controls were negative.

4. Staining with potassium dichromate-silver nitrate gave a positive reaction, indicating the presence of a mercaptide in the metabolites.

5. Synthetic complexes of BSP with cysteine, cystine or glutathione had the same chromatographic mobility as the two naturally occurring metabolites. Amino acids lacking the sulfhydryl group failed to form complexes.

6. It was concluded that BSP is excreted at least in part as a mercaptide with cysteine or the peptide glutathione.

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EFFECT OF HEPATIC DYSFUNCTION ON CIRCULATING LEVELS OF SULFOBROMOPHTHALEIN AND ITS METABOLITES *

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Sulfobromophthalein (BSP) clearance has proved a sensitive index of hepatic function. Although dosage, sampling intervals and techniques of measurement have been modified (1–4), the procedure remains essentially as originally described (5, 6). Basically it consists of the intravenous injection of a standard amount of BSP and determination of residual BSP in the plasma from blood samples collected at prescribed intervals.

Since clearance of BSP is decreased both in hepatocellular dysfunction and biliary obstruction, its determination is of limited usefulness in differentiating these two conditions. Recent studies, however, have shown that BSP is conjugated in the liver and excreted primarily as conjugates into the bile and to a lesser extent into the urine (7–11). Chemical analysis indicates that the dye is conjugated with cysteine and possibly with the peptide, glutathione (12). These metabolites are colorimetrically indistinguishable from free BSP. The demonstration that sulfobromophthalein is conjugated by the liver suggests the following possibilities: *1*) where uptake or hepatic conjugation of BSP is impaired due to parenchymal dysfunction, the circulating dye should remain in the unconjugated form; *2*) in biliary obstruction, where conjugation of the dye proceeds normally but secretion from the liver is impaired, increasing amounts of conjugated dye should appear in the blood. If such is true, the determination of the metabolized BSP, in addition to total BSP, in serum might serve to distinguish impairment of uptake or conjugation from intrahepatic or extrahepatic biliary obstruction.

In this study the levels of free and conjugated BSP were investigated in sera and urine from normal subjects and patients with hepatic disease.

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SUBJECTS AND METHODS

The 87 subjects selected for study consisted of 17 patients with normal liver function, 10 patients in whom liver function tests and clinical work-up (including liver biopsy when feasible) indicated viral hepatitis, 33 patients with portal cirrhosis and varying degrees of hepatic decompensation, 10 patients with extrahepatic obstruction as indicated by liver function studies and confirmed by surgical exploration, and 17 patients initially with normal liver function who were given methyltestosterone (67 mg. per day) for 14 days.

The following liver function tests were carried out in each group: total serum protein and albumin (13); alkaline phosphatase (14) (upper limit of normal in our laboratory is 8 units); one minute and total bilirubin (15) and 45 minute BSP retention (16). In addition, serum glutamic oxalacetic transaminase levels (17) were determined in the subjects treated with methyltestosterone.

The circulating level of BSP metabolites was determined in fasting subjects as follows. Commercial BSP (5 mg. per Kg. body weight) was injected intravenously. Blood was drawn from the opposite arm at specific intervals. The BSP pigments were extracted from 2 ml. of serum [1] with 6 ml. of acetone. Less than 3 per cent of the total pigment remained in the protein fraction of sera containing high concentrations of the metabolites. The acetone extract was taken to dryness *in vacuo* and redissolved in water (approximately 0.2 ml. per ml. of initial serum). A 0.2 ml. aliquot of the solution was chromatographed on Whatman 3 MM paper in an ascending system employing *tert*-butanol-water (1.73:1 v/v) for six to 15 hours. The BSP bands were developed with ammonia fumes. Both major metabolite bands (R_f approximately 0.65 and 0.23) were cut from the strip, pooled and macerated in 25 ml. of 0.05 N NaOH. The mixture was centrifuged for 30 minutes to remove the paper pulp. Duplicate samples of 6 ml. of the supernatant were transferred to an Evelyn cuvette and read against a blank of 0.05 N NaOH at 565 and 620 mμ. The unconjugated BSP band (R_f approximately 0.88) was similarly extracted from the paper and quantified.

[1] Serum could be stored at − 20° C. for several months without affecting the results of the tests.

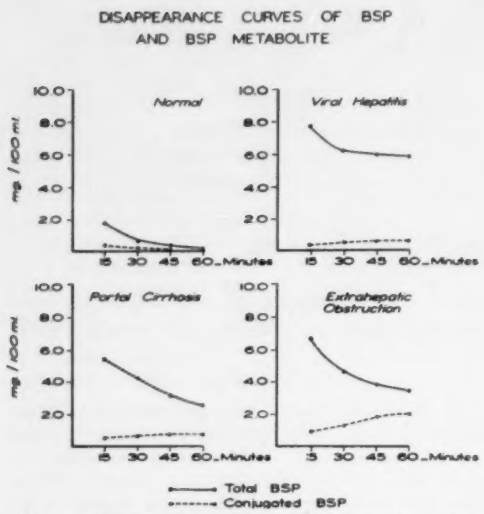

FIG. 1. COMPARISON OF TOTAL AND CONJUGATED SULFOBROMOPHTHALEIN FROM NORMAL SUBJECTS AND FROM PATIENTS WITH VIRAL HEPATITIS, PORTAL CIRRHOSIS AND EXTRAHEPATIC OBSTRUCTION

The percentage of BSP conjugate was calculated according to the formulas:

$$\frac{O.D._{565} - 1.3\,O.D._{620}\text{ (unknown)}}{O.D._{565} - 1.3\,O.D._{620}\text{ (standard)}} \times \text{concentration of standard} = \text{mg. BSP per 100 ml. and}$$

$$\frac{\text{BSP conjugate}}{\text{free BSP} + \text{BSP conjugate}} \times 100 = \text{per cent BSP conjugate.}^{2}$$

The standard deviation obtained after duplicate determinations on 15 different serum samples was 2.1 per cent.

Renal excretion of free and conjugated BSP was determined in several patients from each group as follows. Urine was collected for two hours after injection of the dye. A 30 ml. aliquot was taken to dryness in a rotary evaporator at 40° C. The concentrate was taken up in a minimum volume of distilled water and extracted with three volumes of acetone. The acetone extract was chromatographed and the BSP bands were quantitatively evaluated as described for the serum extracts.

RESULTS

Rate of appearance of BSP and its metabolites

To establish optimal sampling intervals, BSP retention was measured in five patients each from the groups with normal liver function, cirrhosis, acute viral hepatitis and extrahepatic biliary tract obstruction. Measurements were made on blood samples drawn at four 15 minute intervals after injection of the dye (Figure 1).

In the normal subjects BSP was cleared rapidly from the circulation. At the end of 45 and 60 minutes only traces of free and conjugated dye were present in the serum. In the patients with hepatitis, although large amounts of the free dye were retained, the serum contained only small quantities of the conjugated form. In the patients with cirrhosis, the retention of dye was not so pronounced and the amount of conjugated dye was somewhat greater than that found in the patients with viral hepatitis. In the patients with extrahepatic obstruction, however, the amount of conjugated dye increased progressively throughout the test period and was significantly greater than in the other three groups.

The 45 and 60 minute intervals for sampling levels of free and conjugated dye were equally adequate for distinguishing the various types of hepatic dysfunction. Since the major experience in this laboratory has been with the 45 minute BSP test (16), this sampling time was selected for use in this study.

Results of liver function tests

Viral hepatitis (*Table I*). In the patients with viral hepatitis, total BSP retention 45 minutes after injection of the dye was 56.4 ± 2.4 per cent.[3] Of this high percentage of retained dye only 8.0 ± 2.3 per cent was in the conjugated form. All of the subjects were jaundiced. The mean total serum bilirubin concentration was 11.1 mg. per 100 ml. of serum, of which 47.4 per cent was direct bilirubin (presumably bilirubin glucuronide).

Portal cirrhosis (*Table I*). In this group total BSP retention averaged 31.8 ± 2.8 per cent. Of the circulating dye only 16.9 ± 1.5 per cent was in the form of metabolites. Mild jaundice was generally observed. The mean total serum bilirubin concentration was 3.8 ± 1.0 mg. per 100 ml., of which 39.3 per cent was direct bilirubin.

Extrahepatic obstruction (*Table II*). In the patients with extrahepatic obstruction, total BSP

[2] This calculation assumes that the free and conjugated dye have the same molecular extinction coefficient. Accurate determination of the constant for the conjugates must await their availability in crystalline, pure form.

[3] Mean plus or minus standard error.

retention was elevated (40.3 ± 5.0 per cent). In these subjects, however, almost one-half the total circulating BSP (41.1 ± 6.9 per cent) was in the metabolized form. The mean total bilirubin was consistently elevated. Direct bilirubin comprised 36.2 per cent of the total, a value similar to those observed in the first two groups. Serum alkaline phosphatase was notably elevated (32.3 ± 3.4 units per 100 ml.).

Intrahepatic obstruction (*Table II*). In the subjects given methyltestosterone the mean BSP retention was moderately high (19.6 ± 5.0 per cent). As in the group with extrahepatic obstruction a large proportion of the circulating dye

TABLE I

Results of liver function tests in patients with viral hepatitis and portal cirrhosis

Patient	Age	Sex	Total protein	Albumin	Alkaline phosphatase	Bilirubin		Total retained BSP	Conjugated BSP
						Direct	Total		
			Gm./100 ml.		*Units*	*mg./100 ml.*		%	*% of retained BSP*
Viral Hepatitis									
E.V.	31	F	6.5	1.2	10.6	6.0	11.8	44	8
B.T.	25	F	7.2	3.6	3.0	6.1	15.0	47	9
F.S.	24	M	6.6	3.1	9.0	0.9	1.9	51	7
M.H.	30	F	9.2	2.2	11.2	4.7	9.3	54	4
J.B.	24	F	7.1	3.5	10.2	8.1	15.6	55	15
E.D.	33	M	8.3	3.5	5.0	0.8	2.0	58	0
H.I.	40	F	7.7	3.3	8.0	2.2	5.5	61	12
E.G.	62	F	6.5	4.0	13.0	7.3	20.8	65	8
G.R.	32	M	6.4	3.2	9.0	7.3	12.3	67	9
M.B.	58	F	8.0	3.1	5.0	9.2	16.8	62	8
				Mean		5.3	11.1	56.4	8.0
				Standard error of mean		1.0	2.0	2.4	2.3
Portal Cirrhosis									
W.P.	54	M	7.1	3.2	4.1	0.1	0.7	3	4
J.M.	70	M	5.7	3.1	7.7	1.1	1.6	10	36
A.J.	59	M	8.8	4.3	5.8	0.1	1.0	10	15
F.T.	50	M	6.9	4.0	9.6	0.06	0.1	14	33
H.B.	52	F	7.2	2.9	5.6	0.3	1.2	16	23
D.B.	47	M	7.7	2.5	11.3	0.2	0.6	17	49
H.B.	49	M	7.2	3.5	5.6	0.1	0.3	18	36
P.J.	49	F	7.8	2.7	6.0	1.2	3.0	19	9
J.K.	80	M	5.3	3.1	6.8	0.2	0.6	20	4
M.C.	62	M	4.4	3.0	10.1	0.02	0.04	24	27
B.V.	43	F	6.9	1.7	15.9	0.7	1.9	26	15
E.R.	43	M	6.8	2.1	6.8	0.3	0.7	27	24
M.D.	78	F	6.4	2.9	8.5	0.1	0.4	28	20
P.D.	39	M	7.4	1.2	6.2	0.6	1.5	28	21
C.C.	58	F	5.3	3.0	11.0	2.3	5.0	28	14
F.G.	42	M	7.2	2.7	5.9	0.4	1.0	28	14
H.C.	60	F	6.3	3.5	4.1	0.06	0.4	29	17
J.M.	60	M	6.7	3.0	8.0	0.1	0.4	29	6
P.B.	43	F	8.7	4.6	11.0	0.4	1.2	30	14
J.S.	41	M	5.7	1.9	18.6	5.0	13.4	32	21
J.V.	45	M	8.3	2.2	20.0	10.0	28.0	33	9
G.C.	58	F	6.3	1.5	20.0	1.5	3.0	38	10
P.S.	55	F	8.0	3.8	3.8	0.2	0.8	39	0
H.C.	16	F	6.2	2.9	6.0	2.2	6.8	41	21
A.D.	49	M	6.4	2.2	11.1	2.8	7.7	41	9
M.W.	58	F	8.4	2.7	20.0	1.6	5.8	45	20
J.D.	48	M	5.9	2.4	13.0	2.8	4.5	45	13
H.G.	49	F	5.6	1.3	11.0	0.6	2.0	46	7
L.B.	56	F	7.8	3.0	7.4	0.6	1.0	46	17
T.W.	40	F	7.2	1.8	12.8	1.5	5.1	50	11
A.D.	40	F	7.0	2.6	20.0	9.0	18.5	51	12
R.G.	60	F	5.8	2.6	10.3	0.5	1.1	66	18
J.M.	63	M	6.9	3.7	5.8	2.8	6.7	74	8
				Mean		1.5	3.8	31.8	16.9
				Standard error of mean		0.4	1.0	2.8	1.5

TABLE II

Results of liver function tests in patients with extrahepatic and intrahepatic obstruction

Patient	Age	Sex		SGOT*	Protein	Albumin	Alkaline phosphatase	Bilirubin		Total retained BSP	Conjugated BSP
								Direct	Total		
				units/ml.	*Gm./100 ml.*		*units*	*mg./100 ml.*		*%*	*% of retained BSP*
Extrahepatic obstruction											
E.A.	76	M	Common duct stone		6.9	2.5	40	1.2	2.1	17	76
C.T.	64	F	Common duct stone		9.3	2.8	10	1.5	2.5	29	36
J.W.	18	F	Common duct stone		7.1	3.9	18	1.1	2.3	31	38
S.B.	44	M	Stricture of common duct		7.8	4.6	32	2.3	5.0	32	25
B.V.	56	F	Secondary biliary cirrhosis		9.6	4.6	48	0.6	1.0	35	83
D.M.	36	M	Lymphosarcoma with common duct obstruction		7.4	4.7	40	12.0	28.0	35	36
H.J.	46	M	Common duct stone		6.6	2.2	32	3.5	7.3	45	32
J.K.	75	M	Carcinoma of pancreas		6.1	3.2	28	6.4	13.6	48	24
A.M.	69	M	Carcinoma of ampulla of Vater		6.9	2.6	20	5.2	31.3	65	26
A.T.	60	M	Common duct stone		8.0	3.2	55	0.4	1.4	66	35
			Mean					3.4	9.5	40.3	41.1
			Standard error of mean					1.2	3.0	5.0	6.9
Intrahepatic obstruction											
E.B.	33	F		25.0	8.1	4.8	4.2	0.05	0.3	4	Trace
R.R.	25	F		65.0	8.3	4.5	2.8	0.06	0.1	7	25
B.P.	35	F		25.0	8.0	4.1	2.5	0.01	0.0	9	60
J.E.	70	M		40.0	6.6	3.7	3.9	0.09	0.2	11	34
N.P.	58	F		30.0	7.1	3.3	4.6	0.02	0.2	12	16
E.R.	49	F		5.3	6.3	3.4	8.2	0.06	0.1	12	63
E.S.	52	F		25.0	7.5	3.0	5.2	0.09	0.1	13	50
J.S.	80	F		30.0	6.3	3.4	6.9	0.03	0.2	14	36
L.L.	52	F		30.0	8.4	3.9	6.3	0.05	0.2	18	25
M.F.	59	M		65.0	6.2	3.4	3.5	0.07	0.2	20	18
C.D.	74	F		345.0	6.6	3.4	16.7	0.06	0.1	21	46
B.S.	52	F		30.0	7.9	3.8	5.8	0.01	0.3	22	33
N.A.	49	F		75.0	7.4	3.9	5.4	0.04	0.1	24	54
L.L.	83	F		80.0	5.6	3.2	4.1	0.05	0.2	24	62
M.F.	54	M		30.0	6.6	3.7	6.0	0.13	0.3	25	59
E.S.	46	F		110.0	7.8	4.2	7.4	0.13	0.3	26	40
E.P.	71	M		200.0	7.6	4.1	4.0	1.50	2.9	68	30
			Mean					0.1	0.3	19.6	38.9
			Standard error of mean					0.1	0.5	5.0	3.2

* SGOT = serum glutamic oxalacetic transaminase.

(38.9 ± 3.2 per cent) was in the conjugated form. A rise in serum bilirubin concentration occurred in only one of the 17 subjects and a rise in serum alkaline phosphatase levels in two. Only seven of the subjects showed an elevation in serum glutamic oxalacetic transaminase levels.

Statistical significance of differences

Although the percentage of conjugated dye was significantly higher in the cirrhotic subjects than in the patients with viral hepatitis ($p < 0.001$), the mean difference between the two groups was comparatively small. The percentage of conjugate in the groups with extrahepatic and intrahepatic obstruction did not differ significantly ($p = 0.7$). The percentages in both of these groups, however, were approximately five times greater than the percentage of conjugates in the group with hepatitis and twice as high as that found in cirrhosis. Both these differences were highly significant ($p < 0.001$).

Renal excretion of BSP and BSP metabolites (Table III)

The normal subjects excreted small amounts of dye into the urine. In patients with BSP re-

tention induced by hepatitis, cirrhosis or obstruction the excretion of total BSP in the urine was five to 10 times greater than normal. The percentages of conjugated dye in the urine of each group of subjects, however, were not remarkably different and could not serve to differentiate the various disease states from each other or from the normal. In all groups a higher percentage of conjugated BSP was found in the urine than in the serum.

DISCUSSION

Several investigators (18, 19) have noted a time lag between the uptake of BSP by the liver and its eventual secretion into the bile and suggested that clearance of the dye by the liver involves two independent processes, uptake and secretion. The demonstration of conjugates of BSP (7–11) extends this scheme to include uptake, conjugation and secretion. Whether conjugation is the rate-limiting factor in uptake or secretion or whether it represents an intermediary process is not known. Impaired clearance of BSP could result from interference with any one or any combination of these processes.

Since the retained BSP is primarily in the unconjugated form in viral hepatitis, the defect may be either in the mechanism for the uptake or the conjugation of the dye, or both. Because such small amounts of the dye are conjugated to the form normally available for secretion, secretory function could not be evaluated under these circumstances. Subjects with cirrhosis also demonstrated a defect in uptake or conjugation of BSP. The somewhat elevated percentage of circulating metabolites found in these patients may indicate partial impairment of the secretory mechanism.

TABLE III

Appearance of sulfobromophthalein in urine two hours after injection of dye (5 mg. per Kg. body weight)

Group	No. of subjects	Total BSP	Conjugated BSP
		mg./100 ml.	%
Normal	5	2.7 ± 1.1	72.0 ± 13.8
Hepatitis	5	26.2 ± 5.1	52.4 ± 4.5
Cirrhosis	8	21.1 ± 6.5	47.6 ± 7.9
Extrahepatic obstruction	4	13.5 ± 5.6	78.0 ± 5.5
Intrahepatic obstruction	4	11.8 ± 2.6	63.0 ± 7.0

Intrahepatic cholestasis may occur in fatty liver and florid cirrhosis as a result of strangulation of the bile ducts by scar tissue or inflammatory exudate (20). The high percentage of circulating conjugates in the subjects with obstruction of the common bile duct indicates that in this group the mechanisms for uptake and conjugation were functioning but that secretion was impaired. The moderate retention of free dye in these patients could result *1*) directly from some impairment of the mechanism of uptake or conjugation, or *2*) indirectly from impaired secretion resulting in trapping of large amounts of BSP conjugates in the liver, which in turn might cause product inhibition of the conjugating system. Methyltestosterone is known to cause hepatic dysfunction characterized by BSP retention, hyperbilirubinemia, hyperphosphatasemia and cholestasis (21, 22). The high percentage of BSP metabolites in the subjects treated with methyltestosterone suggests that the secretory mechanism is the site of the primary defect responsible for BSP retention. In recent studies of BSP clearance rates, similar conclusions have been suggested (23). In physical obstruction an increase in the circulating level of other substances normally excreted by the liver would be expected. Although this was true in patients with obstruction of the common bile duct, in the subjects treated with methyltestosterone, an elevation of BSP metabolites was apparent long before any rise in serum bilirubin or alkaline phosphatase became evident. This preferential appearance could be entirely a concentration effect. On the other hand, it does suggest that secretion at the cellular level may involve metabolic processes, some of which are specifically inhibited by the steroid, and that this inhibition produces a "metabolic" obstruction.

Determination of BSP and its conjugates may aid in distinguishing hepatic function disorders involving disturbances of uptake and conjugation from those involving secretion or regurgitation of BSP. In the presence of jaundice total BSP retention is almost invariably demonstrable. For this reason it has been suggested that measurement of retention of the dye where hyperbilirubinemia exists contributes little to the evaluation of liver function (24). Differentiation of hepatic function by measurement of the BSP me-

tabolites, however, was possible in the presence or absence of hyperbilirubinemia. Furthermore, the differentiation was still possible in severe liver disease where the ratio of conjugated to free bilirubin proved of little value.

In the subjects given methyltestosterone, BSP retention proved more sensitive than serum bilirubin and serum glutamic oxalacetic transaminase levels in reflecting hepatic dysfunction, and the percentage of BSP conjugates was more sensitive than the serum alkaline phosphatase level in indicating secretory impairment of the liver cell. The sensitivity of the BSP test may depend on administration of large amounts of the dye, sufficient to load the various systems involved in its metabolism. This loading may emphasize slight hepatic functional defects which are not reflected by changes in serum bilirubin and alkaline phosphatase levels. Since BSP is an exogenous substance, alteration of serum levels by its endogenous production, such as occurs with bilirubin, is not a complicating factor. In addition, BSP and bilirubin may reflect different functions of the liver: mercaptide synthesis and secretion by BSP and glucuronide and sulfate formation by bilirubin. The BSP test becomes in part a measure of a specific biochemical reaction in the liver. Under certain circumstances this reaction may be specifically inhibited while other liver functions proceed.[4]

The percentage of conjugated dye in the urine was usually higher than the percentage in the serum of the same subject. Apparently the BSP metabolites are preferentially excreted in the urine, or they may be synthesized by the kidney. The latter possibility is supported by the demonstration of BSP conjugates in the urine of hepatectomized dogs (25).

SUMMARY

The levels of free and conjugated sulfobromophthalein (BSP) were determined in sera and urine from normal subjects and patients with hepatic dysfunction. The results showed that normal subjects conjugate and secrete BSP rapidly; only trace amounts of BSP and BSP metabolites are present in the serum 45 minutes after intravenous injection of a standard dose of the dye. Patients with viral hepatitis and cirrhosis conjugate BSP poorly and retain large amounts of unconjugated dye. Patients with impaired liver function caused by methyltestosterone and extrahepatic biliary obstruction conjugate the dye and accumulate large amounts in the serum. This accumulation was noted before changes in serum bilirubin, alkaline phosphatase and glutamic oxalacetic transaminase were evident. The measurement of the BSP metabolites appears sensitive for differentiating liver defects in uptake and conjugation from those affecting intracellular or extracellular obstruction.

Urinary BSP was mainly in the conjugated form. The percentages of conjugated dye in the urine of each group of subjects were not remarkably different and could not serve to differentiate the various disease states from the normal.

[4] Through the courtesy of Dr. Barbara Billing (London, England), Dr. Leon Schiff (Cincinnati, Ohio), Dr. Owen C. Peck (Palo Alto, California) and Dr. Charbonnier (Paris, France), we had the opportunity of studying serum samples from three patients, all of which failed to show BSP conjugate. In all three specimens direct bilirubin was elevated, indicating formation of bilirubin glucuronide.

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QUANTITATIVE ASPECTS OF THE REACTION BETWEEN INSULIN AND INSULIN-BINDING ANTIBODY

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The presence of insulin-binding antibodies in the serums of insulin-treated subjects has been reported previously (1). With I^{131}-labeled insulin it has been shown that the antigen-antibody complexes do not precipitate, that the binding of insulin to antibody is a reversible process, and that, at constant antibody concentration, the ratio of bound insulin to free insulin is an inverse function of the concentration of insulin (1).

In the present studies, experimental data on the reaction between insulin and insulin-binding antibody are tested against several theoretical models. The results are most compatible with a model composed of a univalent insulin reacting with two distinctly different orders of antibody sites. Data for equilibrium constants, forward and reverse velocity constants and various thermodynamic constants are presented in terms of the model chosen.

METHODS

Antiserums were obtained from insulin-treated diabetic and schizophrenic subjects generally at least 24 hours and frequently many weeks following the last injection of insulin and were stored frozen or at 4° C. for weeks or months prior to use.

Insulin-I^{131} was prepared from crystalline beef insulin[1] with specific activities of 10 to 100 mc. per mg. Methods employed in preparation and in protection of the insulin against extensive radiation damage have been described (2, 3). However, because of the extremely high specific activities of the preparations and the trauma incident to the labeling procedure, storage and dilution, occasionally as much as 10 per cent of the insulin-I^{131} was damaged by the time of use. Recent preparations, with which the bulk of the quantitative data has been obtained, have uniformly contained less than 5 per cent damaged components.

All dilutions were made in water or veronal buffer,[2] 0.1 ionic strength, pH 8.6, to which was added 2 per cent serum albumin to minimize losses of insulin or antibody by adsorption on glassware.

Two types of experiments were performed. In one, henceforth termed "equilibrium state" studies, various relative concentrations of antiserum and insulin, including tracer quantities of insulin-I^{131}, were incubated together at 37° C. The mixtures were allowed to come to equilibrium and were then analyzed for their content of free and bound insulin. No differences in the binding of insulin-I^{131} and unlabeled crystalline insulin could be detected, *i.e.*, at any particular insulin concentration the same degree of binding of insulin-I^{131} was observed whether the insulin present was derived solely from the labeled insulin preparation or was primarily unlabeled insulin with only a tracer amount of insulin-I^{131}. In the other type of experiment, frequently called "rate" studies but herein termed "transient state" studies, insulin and whole or diluted antiserum were each allowed to come to temperature equilibrium at 37° C. and then mixed together. The reaction mixtures were analyzed at intervals to determine the rate of formation of insulin-antibody complexes ("association"). The reverse reaction ("dissociation") was studied by adding high concentrations of nonradioactive insulin to the incubation mixtures; thus, insulin-I^{131} dissociating from preformed insulin-antibody complexes was competitively inhibited from recombination with antibody.

The distribution of insulin-I^{131} between free and bound fractions was determined by paper chromatographic methods which have been described previously (1). In brief, these methods depend on the adsorption of free insulin to the paper at the site of application ("origin") while insulin bound to antibody migrates with the serum proteins. It has been noted previously (1) that during paper electrophoresis at 20° C., in runs lasting 18 to 24 hours, the paper may adsorb some of the insulin dissociating from preformed complexes during the run; this results in a "tailing" of insulin-I^{131} along the path of migration of the complexes so that this procedure does not permit eminently satisfactory separation of the free and bound insulin present initially. However, movement of serum proteins away from the origin, sufficient for resolution of free and bound insulin, can be effected within 20 to 30 minutes by hydrodynamic flow chromatography combined with electrophoresis (1). Since the rates of both forward and reverse reactions are markedly decreased by lowering the temperature, all chromatographic runs were performed in a cold room at 4° C. Under these conditions uncomplexed insulin becomes bound to the paper in a fraction of a minute and generally neither the forward nor the reverse reaction occurs to any significant extent after the mixtures are

[1] We are indebted to Dr. O. K. Behrens and Dr. C. W. Pettinga of the Eli Lilly laboratories for generous supplies of crystalline beef insulin.

[2] No significant differences in binding were detected when duplicate specimens of the same antiserum were diluted in water and in veronal.

applied to the paper strips.[3] In order to maintain reproducible conditions during chromatography of samples at different serum dilutions, 50 μl. of undiluted control plasma was added to all diluted samples at the time of chromatography.

Another method also was used to determine the rates of dissociation of preformed complexes. After incubation of antiserum and insulin-I^{131}, the mixture was added to a source of active "insulinase" (concentrated rat liver homogenate) which is capable of destroying 60 per cent of trace amounts of unbound insulin-I^{131} in the first minute but does not act on bound insulin (4). The rate of destruction of the insulin-I^{131} is therefore limited by the rate of dissociation of the complex. The rate of insulin-I^{131} degradation was evaluated by analysis of aliquots of the mixtures for trichloracetic acid precipitable and non-precipitable radioactivity (4).

The ability of serum to degrade insulin-I^{131} on prolonged incubation at 37° C. has been observed (1). Although many serums are relatively free of such action, some serums are quite troublesome and have to be discarded. Aging and products of hemolysis appear to correlate with the presence of insulin-degrading factors. Whether hemoglobin itself is responsible is not known but since sulfhydryl-containing substances such as H_2S, cysteine and thioglycollate produce similar changes, it appears possible that reduced glutathione, or -SH groups of blood proteins are responsible for the changes observed in the insulin-I^{131}. These alterations are characterized by the appearance of I^{131}-labeled fractions which bind quite firmly to all serum proteins although mainly to α_2-globulin (1); deiodination *per se* is minimal. Damaged fractions produced during the course of preparation of insulin-I^{131} also bind rather indiscriminately to all serum proteins (1–3). Correction for the nonspecific binding of I^{131}-labeled fractions has been made in each experiment by simultaneously running control non-immune plasma samples for evaluation of the damaged moiety in the preparation employed and by electrophoretic analysis for evaluation of "incubation damage." In most experiments these corrections amounted to about 5 per cent of the undamaged insulin-I^{131}. Where damage was significantly in excess of 10 per cent, results are not reported.

Analysis. Since the antigen-antibody complexes under consideration are soluble,[4] direct determination either of the amount of antibody present or of the relative molecular proportions of insulin and antibody in the complexes formed has not been possible. It is necessary, therefore, to consider a number of possible models and to exclude those which are clearly inconsistent with the experimental data. The analysis presented below is employed for this purpose as well as for evaluation of the kinetic data.

The assumption that the law of mass action is applicable to the reactions between antigen and antibody forms the basis of all theoretical considerations which follow.

Equilibrium state studies

Model: Insulin univalent; single order of antibody combining-sites. Let [Ag] be the molar concentration of unbound ("free") insulin, [Ab] the molar concentration of unoccupied antibody combining-sites[5] of a single order,[6] 1 Mole of antibody combining-sites being defined as that quantity of antibody capable of binding 1 Mole of insulin, and $[\overline{AgAb}]$ the molar concentration of bound insulin or occupied antibody combining-sites. Then,

$$[Ag] + [Ab] \underset{k'}{\overset{k}{\rightleftarrows}} [\overline{AgAb}] \qquad 1)$$

$$K = \frac{k}{k'} = \frac{[\overline{AgAb}]}{[Ag][Ab]} \qquad 2)$$

where K is the equilibrium constant and k and k′ are the forward and reverse velocity constants, respectively.

Let [Ab°] denote the molar concentration of total antibody combining-sites[7] so that

$$[Ab^\circ] = [Ab] + [\overline{AgAb}]. \qquad 3)$$

It follows from Equations 2 and 3 that

$$K([Ab^\circ] - [\overline{AgAb}]) = \frac{[\overline{AgAb}]}{[Ag]}.[8] \qquad 4)$$

[3] When antibody concentration is extremely high as in undiluted serums from insulin-resistant subjects, the forward reaction may continue to a significant extent until the plasma proteins have migrated away from the origin, which may take several minutes. In such cases antibody may actually elute a fraction of the insulin previously adsorbed to the paper. For these reasons and also to slow down the rate of the forward reaction to measurable levels, serums of high antibody titer were always diluted appreciably for transient state studies. Although, after *prolonged* chromatography, slight elution of insulin, adsorbed to paper by the solvent, can be detected as a broadening of the peak at the origin, the absence of any significant elution under the conditions employed here has been demonstrated in experiments with undiluted control plasma and undamaged insulin-I^{131} preparations. In these cases there is no detectable radioactivity migrating away from the peak at the origin. The absence of any significant loss of bound insulin-I^{131} from the complexes by dissociation and adsorption to paper during chromatographic development has also been established by the observation that over 99 per cent of trace insulin-I^{131} migrates with the serum proteins in serums with high antibody titers.

[4] Repeated attempts to demonstrate evidence of precipitation after prolonged periods of incubation and refrigeration have been unsuccessful.

[5] Inasmuch as the valency of insulin-binding antibody is not known, the analysis is best formulated in terms of antibody sites rather than in terms of antibody molecules with an assumed valency.

[6] An order of antibody combining-sites is defined, for the purpose of the present study, as a group in which the individual sites are not necessarily identical chemically, but nevertheless exhibit sufficiently small variations in affinity for the antigen as to be indistinguishable under the experimental conditions.

[7] Thus, [Ab°] = valency of antibody × molar concentration of antibody.

[8] This form is similar to that employed by Scatchard (5) in a study of ion-binding by proteins.

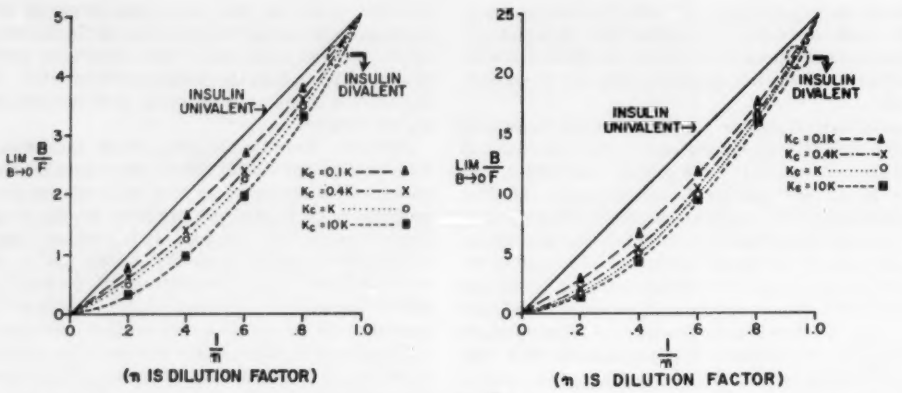


FIG. 1. EFFECT OF DILUTION OF ANTISERUM ON $\underset{B\to 0}{\text{LIM}}\ \frac{B}{F}$ FOR UNIVALENT AND DIVALENT INSULIN MODELS AND FOR INITIAL $\frac{B}{F}$ VALUES OF 5 (LEFT) AND 25 (RIGHT)

The higher the initial $\frac{B}{F}$ value, the greater the deviation from a straight line for the lower K_c/K ratios.

For convenience, let B and F represent the molar concentrations of bound insulin ($[\overline{AgAb}]$) and free insulin ($[Ag]$), respectively. Equation 4 may then be rewritten,

$$\frac{B}{F} = K([Ab^\circ] - B). \qquad 5)$$

The ratio of bound insulin to free insulin is, therefore, a linear function of the concentration of bound insulin and a plot of $\frac{B}{F}$ vs. B yields a straight line with a slope of $-K$ and an ordinate intercept of $K[Ab^\circ]$.

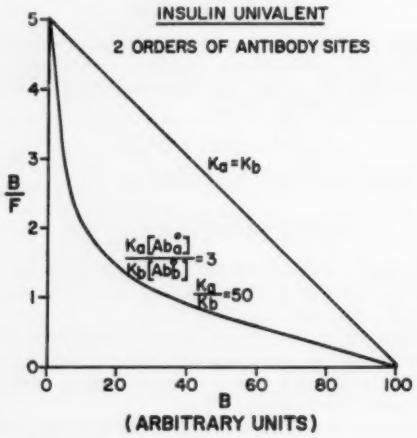


FIG. 2. SCHEMATIC PLOTS OF $\frac{B}{F}$ VS. B FOR UNIVALENT INSULIN REACTING WITH TWO ORDERS OF ANTIBODY COMBINING-SITES

Two curves are shown, one for $K_a = K_b$, the other for a 50-fold difference in K_a and K_b and reciprocal 17-fold difference in antibody concentration (see text). Almost any shaped curve, concave upwards, is possible for this model.

The total concentration of antibody combining-sites is determined either from the values for K and $K[Ab^\circ]$ or from the intercept with the horizontal axis, since $[Ab^\circ] = B$ at $\frac{B}{F} = 0$.

When insulin concentrations are very low relative to $[Ab^\circ]$, B approaches zero and Equation 5 becomes

$$\underset{B\to 0}{\text{Lim}}\ \frac{B}{F} = K[Ab^\circ]. \qquad 6)$$

If the concentration of antibody is lowered by serial dilution of the antiserum and n is the dilution factor,

$$\underset{B\to 0}{\text{Lim}}\ \frac{B}{F} = K\frac{[Ab^\circ]}{n}. \qquad 7)$$

$\underset{B\to 0}{\text{Lim}}\ \frac{B}{F}$ is then directly proportional to $\frac{1}{n}$ (Figure 1).

Model: Insulin univalent; two or more orders of antibody combining-sites. If insulin is univalent and there are two distinct orders of antibody combining-sites (Ab_a and Ab_b), each with characteristic forward and reverse velocity constants, then,

$$[Ag] + [Ab_a] \underset{k_{a'}}{\overset{k_a}{\rightleftarrows}} [\overline{AgAb_a}] \qquad 8a)$$

$$[Ag] + [Ab_b] \underset{k_{b'}}{\overset{k_b}{\rightleftarrows}} [\overline{AgAb_b}] \qquad 8b)$$

and by rearrangements similar to those of the preceding section, it follows that

$$\frac{B}{F} = K_a([Ab^\circ_a] - B_a) + K_b([Ab^\circ_b] - B_b) \qquad 9)$$

where $B = B_a + B_b = [\overline{AgAb_a}] + [\overline{AgAb_b}]$, and K_a and K_b are the respective equilibrium constants.

A plot of $\frac{B}{F}$ vs. B (Figure 2) is a curve, concave upwards, the departure from linearity of which depends on the relative values of the K's and $[Ab^o]$'s. Here the ordinate intercept is $K_a[Ab^o_a] + K_b[Ab^o_b]$. If the K's are quite similar, the curve will approximate a straight line and it may not be possible to determine the individual K's; the mean K will approximate that obtained using Equation 5. If the K's differ significantly, solutions for the two K's and the two $[Ab^o]$'s may be obtained either *1)* by curve-fitting with assumed trial values for the K's and $[Ab^o]$'s or *2)* by substituting experimental values for $\frac{B}{F}$ and $(B_a + B_b)$ into Equation 9. Since Equation 9 contains seven variables, only two of which $\left(\frac{B}{F}$ and $B_a + B_b\right)$ are determined by each point, a minimum of five points is required for solution. The values at the ordinate intercept (B = 0) and at the abscissa intercept ($B = B_a + B_b = [Ab^o_a] + [Ab^o_b]$) provide two points so that values for at least three other points along the curve must be employed. The method of curve-fitting has been found to be more convenient (see Appendix A).

On dilution of the antiserum, at very low insulin concentrations, the ratio $\lim_{B\to 0} \frac{B}{F}$ will again be directly proportional to $\frac{1}{n}$ (as in Figure 1), according to the following equation:

$$\lim_{B\to 0} \frac{B}{F} = K_a \frac{[Ab^o_a]}{n} + K_b \frac{[Ab^o_b]}{n} = \frac{K_a[Ab^o_a] + K_b[Ab^o_b]}{n}. \quad 10)$$

Similar considerations hold for more than two orders of antibody combining-sites.

It is evident that these formulations do not discriminate between a single antibody molecule with more than one order of combining-sites (divalent or multivalent antibody) and different antibody molecules each with a single combining-site of a distinct order. In itself, this distinction is not pertinent to the determination of the equilibrium constants and total binding capacities of the different orders of antibody sites. Nevertheless, some reservation must be held in the case that the two different orders of antibody sites are the two sites on divalent antibody molecules, for then the two equations, 8a and 8b, may not be independent; *i.e.*, because of steric or other restrictions, a complexed antigen molecule may interfere with entry of a second antigen molecule, particularly if the combining-sites on the antibody molecule are closely spaced.

Model: Insulin divalent; one or more orders of antibody combining-sites. If a single molecule of insulin is able to complex with two antibody combining-sites and if all antibody combining-sites are of the same order, the following reactions obtain,

$$[Ag] + [Ab] \underset{k'}{\overset{k}{\rightleftarrows}} [\overline{AgAb}] \quad 11)$$

$$[\overline{AgAb}] + [Ab] \underset{k_c'}{\overset{k_c}{\rightleftarrows}} [\overline{AgAb_2}]. \quad 12)$$

The formulation is complete if the two antigenic sites are identical. If they differ, two sets of equations, 11 and 12, are required to take into account the different possible sequences; if, in addition, cross reaction of each antigenic site with antibody to the other site can also occur, description of all possible sequences would involve four sets of paired consecutive reactions. While these possibilities are of interest they would not alter the conclusions derived from the development which follows and will not be analyzed further here. From Equations 11 and 12:

$$K = \frac{k}{k'} = \frac{[\overline{AgAb}]}{[Ag][Ab]} \quad 13)$$

$$K_c = \frac{k_c}{k_c'} = \frac{[\overline{AgAb_2}]}{[\overline{AgAb}][Ab]}. \quad 14)$$

Since $[Ab] = [Ab^o] - [\overline{AgAb}] - 2[\overline{AgAb_2}]$ and $B = [\overline{AgAb}] + [\overline{AgAb_2}]$, Equations 13 and 14 may be rewritten, after transposition,

$$\frac{[\overline{AgAb}]}{[Ag]} = K([Ab^o] - B - [\overline{AgAb_2}]) \quad 15)$$

$$\frac{[\overline{AgAb_2}]}{[Ag]} = KK_c([Ab^o] - B - [\overline{AgAb_2}])^2 \quad 16)$$

and from the sum of Equations 15 and 16,

$$\frac{B}{F} = K([Ab^o] - B - [\overline{AgAb_2}]) + KK_c([Ab^o] - B - [\overline{AgAb_2}])^2. \quad 17)$$

The ratio, $\frac{[\overline{AgAb_2}]}{[\overline{AgAb}]}$, will decrease progressively with increase in insulin concentration. A plot of $\frac{B}{F}$ vs. B is a curve, which is concave upwards, as in Figure 3. The

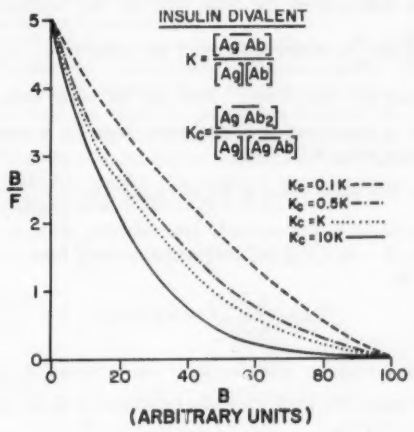


FIG. 3. SCHEMATIC PLOTS OF $\frac{B}{F}$ VS. B FOR DIVALENT INSULIN REACTING WITH A SINGLE ORDER OF ANTIBODY COMBINING-SITES AT DIFFERENT $\frac{K_c}{K}$ RATIOS

ordinate intercept is $K[Ab^o] + KK_e[Ab^o]^2$, and $[Ab^o]$ is given by the intercept with the horizontal axis. It is evident that such curves may not readily be distinguishable from those shown in Figure 2. However, a distinction between the two models is available from the behavior of $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ on dilution of the antiserum. At low insulin concentrations, Equation 17 becomes

$$\underset{B\to 0}{\text{Lim}} \frac{B}{F} = K[Ab^o] + KK_e[Ab^o]^2 = K[Ab^o](1 + K_e[Ab^o]). \quad 18a)$$

If the antiserum is diluted by the factor n,

$$\underset{B\to 0}{\text{Lim}} \frac{B}{F} = \frac{K[Ab^o]}{n} + KK_e\frac{[Ab^o]^2}{n^2} = K\frac{[Ab^o]}{n}\left(1 + K_e\frac{[Ab^o]}{n}\right) \quad 18b)$$

and $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ decreases more rapidly than $\frac{1}{n}$ (Figure 1). K and K_e can be determined from the values for $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ at any two dilutions if $[Ab^o]$ is determined from data plotted as in Figure 3.

It is necessary to consider further the relationships $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ vs. $\frac{1}{n}$ and $\frac{B}{F}$ vs. B. Firstly, it is evident from Equation 18b that, regardless of the relative magnitudes of K and K_e, if $K_e[Ab^o] \gg 1$, the ratio will vary, not with $\frac{1}{n}$ but with $\frac{1}{n^2}$. Conversly, when $K_e[Ab^o] \ll 1$, a linear relationship between $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ and $\frac{1}{n}$ is closely approximated. In the latter event, deviation from a linear relationship might escape detection without excluding thereby the possible divalency of insulin.

The influence of the ratio, $\frac{K_e}{K}$, on the relationship $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ vs. $\frac{1}{n}$ is shown in Figure 1 for initial $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ values of 5 and 25, respectively. Even for the lower initial $\frac{B}{F}$ value, a significant deviation from linearity is readily apparent when $K_e \geqq 0.4K$.

For any given value of K_e, the ratio, $\underset{B\to 0}{\text{Lim}} \frac{[\overline{AgAb_2}]}{[\overline{AgAb}]}$, is proportional to the antibody concentration since, in the region $B \to 0$, $[Ab] \cong [Ab^o]$ and therefore, from Equation 14,

$$\underset{B\to 0}{\text{Lim}} \frac{[\overline{AgAb_2}]}{[\overline{AgAb}]} = K_e[Ab^o]. \quad 19)$$

It is important to note that since the curvature of the $\frac{B}{F}$ vs. B curve (Figure 3) increases with increase in the ratio, $\frac{K_e}{K}$, a markedly curvilinear relationship for $\frac{B}{F}$ vs. B and an apparently straight line relationship for $\underset{B\to 0}{\text{Lim}} \frac{B}{F}$ vs. $\frac{1}{n}$ are incompatible with a model requiring more than a single valency for insulin.

It may also be observed that a reasonable restriction (though not essential to the analysis) for the relationship of K_e to K is that $K_e \leqq K$, since it appears unlikely either that a second antibody molecule is more easily complexed than is the first or that the divalent form of the antigen-antibody complex, $[\overline{AgAb_2}]$, releases an antibody molecule any less readily than does the univalent form $[\overline{AgAb}]$. The reverse situation would seem to be the more likely in view of the possibility that through steric or other restrictions, the antibody molecules would interfere with each other's attachment onto the relatively small insulin molecule.

It is readily shown that if insulin is multivalent and there is a single order of antibody sites,

$$\underset{B\to 0}{\text{Lim}} \frac{B}{F} = K[Ab^o] + KK_e[Ab^o] + KK_eK_d[Ab^o]^3 + \cdots \quad 20)$$

Whether or not there are, in addition, several orders of antibody combining-sites, multivalency of antigen will produce deviations from the straight line of Figure 1 on dilution of the antiserum.

Transient state studies

Model: Insulin univalent. The case of two orders of antibody combining-sites only is presented. From classical chemical kinetics, for the opposing first and second order reactions of Equations 8a and b,

$$\frac{d[\overline{AgAb_a}]}{dt} = k_a[Ag][Ab_a] - k'_a[\overline{AgAb_a}] \quad 21)$$

$$\frac{d[\overline{AgAb_b}]}{dt} = k_b[Ag][Ab_b] - k'_b[\overline{AgAb_b}]. \quad 22)$$

If now, $[Ab^o] \gg [Ag^o]$, where $[Ag^o]$ is the initial concentration of insulin, the second order forward reactions in Equations 8a and b become pseudo first order, since $[Ab_a] \cong [Ab^o_a]$ and $[Ab_b] \cong [Ab^o_b]$ throughout the course of the reaction. Therefore, under conditions in which only tracer quantities of insulin-I^{131} are added to antiserum, Equations 21 and 22 become, with the terminology previously employed,

$$\frac{dB_a}{dt} = k_a[Ab^o_a]F - k'_aB_a \quad [Ab^o_a] \gg [Ag^o], \quad 23)$$

$$\frac{dB_b}{dt} = k_b[Ab^o_b]F - k'_bB_b \quad [Ab^o_b] \gg [Ag^o]. \quad 24)$$

The sum of Equations 23 and 24 yields

$$\frac{dB}{dt} = (k_a[Ab^o_a] + k_b[Ab^o_b])F - (k'_aB_a + k'_bB_b), \quad 25)$$

where, as before, $B = B_a + B_b$.

Since initially there is no B_a or B_b present and all the insulin is in the free form, at zero time Equation 25 reduces to

$$\left(\frac{dB}{dt}\right)_{t=0} = (k_a[Ab^o_a] + k_b[Ab^o_b])[Ag^o]^9 \quad 26)$$

[9] Since at zero time, $[Ab_a] = [Ab^o_a]$ and $[Ab_b] = [Ab^o_b]$, Equation 26 is applicable regardless of the initial concentrations of insulin.

where $[Ag^o]$ may be taken as 1.0 or 100 per cent. Thus, the zero time slope of B vs. t as given by Equation 26 is the fraction or per cent $[Ag^o]$ converted to B per unit time.

If, at any time during the reaction, further reaction between insulin-I^{131} and Ab is inhibited, dissociation of the preformed complexes may be expressed by the following:

$$\frac{dB_a}{dt} = -k'_aB_a$$

$$\frac{dB_b}{dt} = -k'_bB_b \qquad 27)$$

which become, on integration,

$$B_a = \overline{B}_ae^{-k'_at}$$

$$B_b = \overline{B}_be^{-k'_bt} \qquad 28)$$

where $\overline{B}_a$ and $\overline{B}_b$ are the concentrations of bound insulin in the two complexes, respectively, at the time when further combination and reassociation of insulin-I^{131} and antibody are first inhibited. Therefore,

$$B_t = \overline{B}_ae^{-k'_at} + \overline{B}_be^{-k'_bt} \qquad 29)$$

where B_t is the concentration of insulin remaining bound at time t, and t is time elapsed following inhibition of combination or reassociation.

By resolving the two-component curve of Equation 29 into its separate exponential components (one of which represents a more slowly dissociating, the other a more rapidly dissociating complex), values for k'_a, k'_b, $\overline{B}_a$ and $\overline{B}_b$ are obtained. If the reaction between insulin and antibody is allowed to reach equilibrium prior to inhibition, then $\lim_{B\to 0} \frac{\overline{B}_a}{F} = K_a[Ab^o_a]$ and $\lim_{B\to 0} \frac{\overline{B}_b}{F} = K_b[Ab^o_b]$ (cf. Equations 6 and 8). Thus the values for each $K[Ab^o]$ may be determined from "dissociation" rate studies and compared with values obtained from equilibrium state analyses $\left(\frac{B}{F} \text{ vs. } B\right)$.

Also, since from Equation 2,

$$K_a[Ab^o_a] = \frac{k_a[Ab^o_a]}{k'_a} \qquad 30)$$

$$K_b[Ab^o_b] = \frac{k_b[Ab^o_b]}{k'_b} \qquad 31)$$

$k_a[Ab^o_a]$ and $k_b[Ab^o_b]$ can be calculated individually from the values for k'_a, k'_b, $K_a[Ab^o_a]$ and $K_b[Ab^o_b]$ and compared with the sum, $k_a[Ab^o_a] + k_b[Ab^o_b]$, as determined from the zero time slopes of the forward reaction at low insulin concentrations, employing Equation 26.

If, following the addition of tracer quantities of insulin-I^{131} to antiserum, the reaction mixture is analyzed at intervals for free insulin and for bound insulin in each of the complexes B_a and B_b, another approach may be employed for the determination of the $k[Ab^o]$'s and the k''s. The transient state equations may be written:

$$\frac{dF}{dt} = -(k_a[Ab^o_a] + k_b[Ab^o_b])F + k'_aB_a + k'_bB_b \qquad 32)$$

$$\frac{dB_a}{dt} = k_a[Ab^o_a]F - k'_aB_a \qquad 33)$$

$$\frac{dB_b}{dt} = k_b[Ab^o_b]F - k'_bB_b \qquad 34)$$

where Equations 33 and 34 are identical with Equations 23 and 24, respectively.

Solution of these equations for F, B_a and B_b as a function of time, t, employing the boundary conditions that at $t = 0$; $F = 1$, $B_a = B_b = 0$ and at $t = \infty$; $F_{(eq)} + B_{a(eq)} + B_{b(eq)} = 1$, where $F_{(eq)}$, $B_{a(eq)}$ and $B_{b(eq)}$ are the respective equilibrium values, yields;

$$F = F_{(eq)} - (r_1 + s_1)e^{-\lambda_1 t} - (r_2 + s_2)e^{-\lambda_2 t} \qquad 35)$$

$$B_a = B_{a(eq)} + r_1e^{-\lambda_1 t} + r_2e^{-\lambda_2 t} \qquad 36)$$

$$B_b = B_{b(eq)} + s_1e^{-\lambda_1 t} + s_2e^{-\lambda_2 t} \qquad 37)$$

where

$$\lambda_{1,2} = \tfrac{1}{2}\Big((k'_a + k'_b + k_a[Ab^o_a] + k_b[Ab^o_b]) \mp [(k'_a + k'_b + k_a[Ab^o_a] + k_b[Ab^o_b])^2 - 4(k'_ak'_b + k'_ak_b[Ab^o_b] + k'_bk_a[Ab^o_a])]^{\frac{1}{2}}\Big),$$

and

$$r_1 = -\frac{B_{a(eq)}\lambda_2 - k_a[Ab^o_a]}{\lambda_2 - \lambda_1} \qquad r_2 = \frac{B_{a(eq)}\lambda_1 - k_a[Ab^o_a]}{\lambda_2 - \lambda_1}$$

$$s_1 = -\frac{B_{b(eq)}\lambda_2 - k_b[Ab^o_b]}{\lambda_2 - \lambda_1} \qquad s_2 = \frac{B_{b(eq)}\lambda_1 - k_b[Ab^o_b]}{\lambda_2 - \lambda_1}.$$

If $B_{a(eq)}$ and $B_{b(eq)}$ are determined as described in the previous section, a plot of F vs. t (Equation 35) is sufficient for the evaluation of the individual $k[Ab^o]$'s and k''s, since resolution of the curve into its exponential components will provide the necessary values of λ_1, λ_2, $(r_1 + s_1)$ and $(r_2 + s_2)$. However, individual curves for B_a and B_b as a function of time may be obtained by withdrawing samples of the reaction mixture at various times and analyzing for the amount of bound insulin in each of the two complexes according to the procedures described in relation with Equations 28 and 29. The k''s determined in each of these "dissociation" curves and the $k[Ab^o]$'s determined from the zero time slopes of B_a vs. t and B_b vs. t serve to check the values obtained from the analysis of F vs. t according to Equation 35.

Effect of dilution of antiserum. On dilution of antiserum, $[Ab^o_a]$ and $[Ab^o_b]$ are reduced by the same factor. Therefore, at tracer concentrations of insulin, dilution of antiserum does not alter the proportion between the more slowly and the more rapidly dissociating complexes.

Model: Insulin divalent or multivalent. At tracer concentrations of insulin, the transient state equations may be written, from Equations 11 and 12,

$$\frac{dF}{dt} = -k[Ab^o]F + k'[\overline{AgAb}] \qquad 38)$$

$$\frac{d[\overline{AgAb}]}{dt} = -k'[\overline{AgAb}] - k_c[Ab^o][\overline{AgAb}] + k[Ab^o]F + k'_c[\overline{AgAb_2}] \qquad 39)$$

$$\frac{d[\overline{AgAb_2}]}{dt} = -k'_c[\overline{AgAb_2}] + k_c[Ab^o][\overline{AgAb}] - k_d[Ab^o][\overline{AgAb_2}] + k'_d[\overline{AgAb_3}] \qquad 40)$$

$$\frac{d[\overline{AgAb_3}]}{dt} \ldots.$$

If $[\overline{AgAb}]$ is denoted by a, and $[\overline{AgAb_2}]$ by b, solution (in the case of divalent insulin) of Equations 38 through

40 for F, a and b as a function of time, employing the boundary conditions that at $t = 0$; $F = 1$, $a = b = 0$ and at $t = \infty$; $F_{(eq)} + a_{(eq)} + b_{(eq)} = 1$, yields

$$F = F_{(eq)} + r_1e^{-\lambda_1 t} + r_2e^{-\lambda_2 t} \quad 41)$$

$$a = a_{(eq)} - (r_1 + s_1)e^{-\lambda_1 t} - (r_2 + s_2)e^{-\lambda_2 t} \quad 42)$$

$$b = b_{(eq)} + s_1e^{-\lambda_1 t} + s_2e^{-\lambda_2 t} \quad 43)$$

where

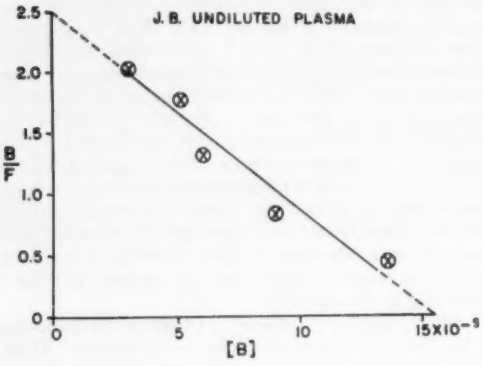
$$\lambda_{1,2} = \tfrac{1}{2}\Big((k[Ab^o] + k_c[Ab^o] + k' + k'_c) \mp [(k[Ab^o] + k_c[Ab^o] + k' + k'_c)^2 - 4(k'k'_c + k'_ck[Ab^o] + kk_c[Ab^o]^2)]^{\frac{1}{2}}\Big)$$

and

$$s_1 = -\frac{\lambda_2 b_{(eq)}}{\lambda_2 - \lambda_1} \qquad r_1 = \frac{\lambda_2(1 - F_{(eq)}) - k[Ab^o]}{\lambda_2 - \lambda_1}$$

$$s_2 = \frac{\lambda_1 b_{(eq)}}{\lambda_2 - \lambda_1} \qquad r_2 = -\frac{\lambda_1(1 - F_{(eq)}) - k[Ab^o]}{\lambda_2 - \lambda_1}.$$

The characteristics of "dissociation" curves will allow for identification of $\overline{AgAb}$ and $\overline{AgAb_2}$ with the slowly and rapidly dissociating complexes. If $\overline{AgAb}$ dissociates less rapidly than $\overline{AgAb_2}$ (*i.e.*, $k'_c > k'$), the slope of the dissociation curve will become steeper with time (or, if the rates differ greatly, say by a factor of 10 or more, the curve will appear to approximate a single exponential component). Only if the converse is true (*i.e.*, $k' > k'_c$) can the initial segment of the dissociation curve have the sharpest slope.[10] Unlike the case of univalent insulin reacting with two orders of antibody sites, the relative amounts of "univalent" and "divalent" complexes are not obtained simply from the extrapolation of the final component to zero time since the amounts in a and b at any time, t, following inhibition of recombination are described by the following equations:



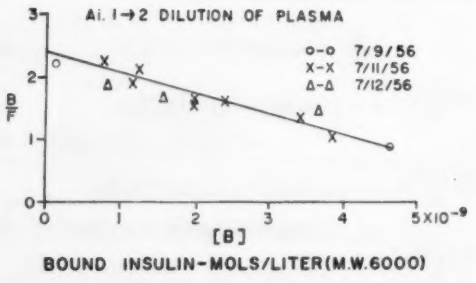


FIG. 4. $\frac{B}{F}$ VS. B CURVES IN J. B. AND AI. APPROXIMATE A STRAIGHT LINE

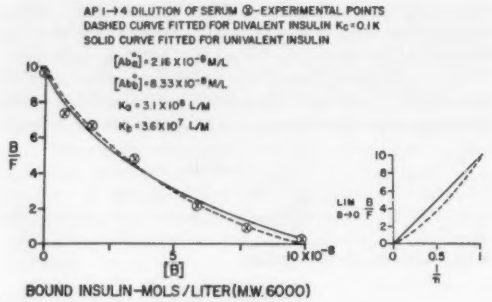


FIG. 5. $\frac{B}{F}$ VS. B DATA (LEFT) FITTED FOR UNIVALENT AND DIVALENT INSULIN MODELS IN SUBJECT A. P. THEORETICAL CURVES OF $\frac{B}{F}$ VS. $\frac{1}{N}$ IN SAME SUBJECT (RIGHT) FOR BOTH MODELS

Experimental $\frac{B}{F}$ vs. $\frac{1}{n}$ curve for A. P. shown in Figure 7.

$$\frac{db}{dt} = -k'_cb \quad 44)$$

$$\frac{da}{dt} = k'_cb - k'a \quad 45)$$

$$b = \bar{b}e^{-k'_ct} \quad 46)$$

$$a = \bar{a}e^{-k't} + \frac{k'_c\bar{b}}{k' - k'_c}(e^{-k'_ct} - e^{-k't}) \quad 47)$$

where $\bar{a}$ and $\bar{b}$ are the amounts of a and b, respectively, present at the time recombination is first inhibited. The concentration of bound insulin-I^{131} remaining at any time is, of course, the sum, $a + b$. If the rates of dissociation of the two complexes are markedly different, the relative amounts of "univalent" and "divalent" complexes are *approximately* given by the coefficients of the two exponential terms in the expression for $a + b$.

Effect of dilution of antiserum. It follows from Equation 19 that the decrease in antibody concentration resulting

[10] For the condition $k' > k'_c$, the initial slope may be sharper than, the same as, or shallower than the final slope, depending on the relative magnitudes of the rates and on the amounts in each form of the complex at the onset of dissociation.

from dilution of the antiserum will be accompanied by a proportionate decrease in the ratio $\frac{[AgAb_2]}{[AgAb]}$ at tracer concentrations of insulin. The relative contributions of the two complexes to the total bound insulin at equilibrium will then differ at different serum dilutions, the ratio decreasing with dilution.

RESULTS

Equilibrium state experiments

In a few cases, plots of $\frac{B}{F}$ vs. B could reasonably be fitted by a single straight line but the data available in these cases were incomplete (Figure 4). In most antiserums a curvilinear relationship was demonstrable and it was evident that a univalent insulin reacting with a single order of homogeneous antibody combining-sites would not suffice to account for the data. Moreover, the shapes of several of the curves were such that if observations had been less complete, particularly for values near one or the other axis, a straight line might have appeared to provide a satisfactory fit, which suggests that the linear relationship in Figure 4 was probably more apparent than real.

In some cases, the experimental data could be fitted almost equally well by assuming either a univalent insulin reacting with two different orders of antibody combining-sites or a divalent insulin reacting with a single order of antibody combining-sites (Figure 5). These were cases in which the slope of $\frac{B}{F}$ vs. B was not particularly steep for low values of B. For cases in which a steep slope in the region of low B was observed (*e.g.*, D. C. and Pr., Figure 6) no fit was possible for the case of a divalent insulin and a single order of combining-sites even if K_e was assumed to be very much greater than K;[11] however, a

[11] This does not, of course, exclude the cases of divalent or multivalent insulin reacting with two or more orders of antibody combining-sites.

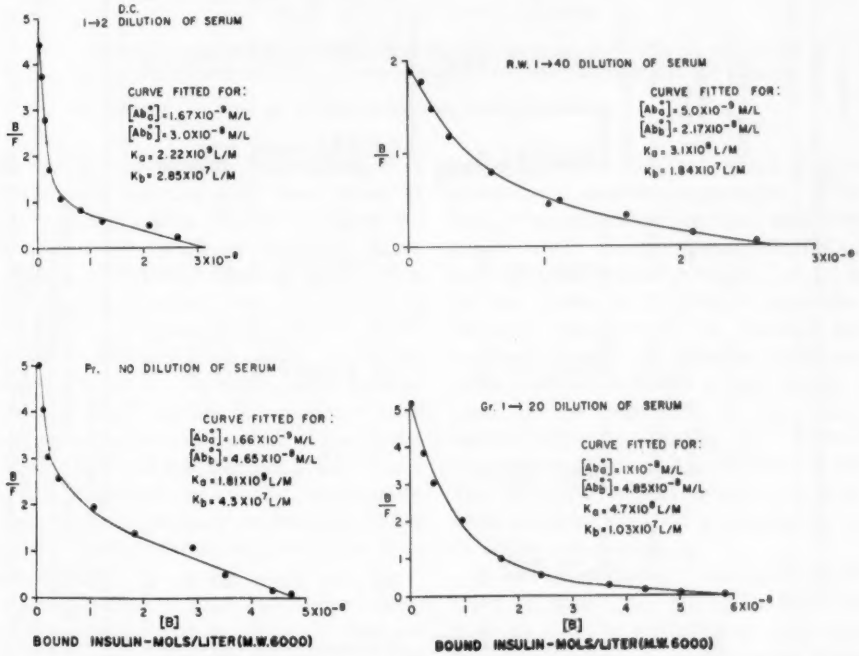


FIG. 6. $\frac{B}{F}$ vs. B CURVES FOR NONRESISTANT SUBJECTS D. C. AND PR. AND RESISTANT SUBJECTS R. W. AND GR. FITTED FOR UNIVALENT INSULIN REACTING WITH TWO ORDERS OF ANTIBODY COMBINING-SITES

Points are experimentally determined values. Curves are theoretical, fitted for values as indicated.

TABLE I

Data pertaining to insulin-binding capacities, equilibrium constants, forward and reverse velocity constants and insulin dosage

Subject	Serum dilution studied	K_a	$[Ab^o_a]$	K_b	$[Ab^o_b]$	$[Ab^o_a + Ab^o_b]$ In undiluted serum		k'_a Observed from dissociation rate studies	k'_b Observed from dissociation rate studies	k_a Calculated	k_b Calculated	$(k_a[Ab^o_a] + k_b[Ab^o_b])$ Calculated	$(k_a[Ab^o_a] + k_b[Ab^o_b])$ Observed from association rate studies	Daily insulin dosage*
		L./Mole	*Moles/L.*	*L./Mole*	*Moles/L.*	*Moles/L.*	*units/L.*	*min.*$^{-1}$	*min.*$^{-1}$	*L./Mole/min.*	*L./Mole/min.*	*%/min.*	*%/min.*	
Insulin-resistant subjects														
1. J. Ha.	1 : 2	1.3×10^{8}†	450×10^{-10}	2.5×10^{7}	127×10^{-9}	3.4×10^{-7}	57	21×10^{-4}	23×10^{-2}	2.7×10^{5}	58×10^{5}	14.9‡	17.5‡	120 U
2. L. J.	1 : 10	23	33	4.8	33	3.6	60							480 U
3. A. P.	1 : 4	3.1	216	3.6	83	4.2	70	18	9.0	5.6	32	28	20	200 U
4. E. M.	1 : 4	2.5	183	1.6	140	6.4	106	10.7	5.8	2.7	9.3	12.5	12.5	150 U
5. L. S.	1 : 50					7.3	122							190 U
	1 : 10	4.8	33	2.1	75	7.8	132	93	28	45	59	46		
6. R. W.	1 : 40	3.1	50	1.8	22	10.8	180	6.5	3.4	2.0	6.1			500 U
7. Gr.	1 : 20	4.7	100	1.0	49	11.7	195							145 U
8. M. L.	1 : 4					13	210							150 U
9. M. S.	1 : 100	12	33	20	13.3	16.6	270							§
10. Ha.	1 : 20					30	500							500 U
11. Ke.	1 : 10					45	750							‖
12. Pl.	1 : 10					280	4,700							¶
Nonresistant subjects**														
13. A. R.	1 : 2	24	5	17	2.3	0.057	0.95	33	6.5	79	110	2.9	6.3	orinase (20 U)††
14. S. B.	1 : 2	180	1.7	46	3.5	0.073	1.2							orinase (25 U)
15. C. L.	1 : 2					0.020	0.33							orinase (27 U)
16. Le.	1 : 2			46	2.5	0.050	0.83							30 U
17. Fa.	1 : 2	20	5.0	11	3.7	0.084	1.4							orinase (15 U)
18. Ru.	1 : 2	168	0.83	30	3.0	0.061	1.0	5.2	17	87	510	16	18	10 U
19. J. B.	undil.			16	15	0.15	2.5 (extrapolated value)							10 U
20. M. D.	1 : 2	12	5.0	4.6	8.7	0.18	3.0	8.2	6.9	9.8	32	2.8	2.9	orinase (10 U)
21. A. C.	1 : 2	24	6.7	7.4	15	0.31	5.2							orinase (15 U)
22. Mo.	1 : 6					0.33	5.5	39	35					50 U
23. Pr.	undil.	18	17	4.3	47	0.49	8.1	81	17.5	145	75	37	30	0 (insulin shock therapy)
24. H. R.	1 : 2	24	6.7	4.3	18	0.57	9.5							orinase (15 U)
25. D. C.	1 : 2	22	17	2.9	30	0.63	10.5	23	4.6	51	13	4.7	14.6	orinase (15 U)
	1 : 4					0.58	9.7							
26. H. L.	1 : 10	64	8.3	5.7	12.5	1.3	22	4.0	3.8	26	22	2.8		0 (55 U)
	1 : 6					1.4								
	1 : 4					1.5								
27. L. Sy.	1 : 2	125	1.7	3.5	67	1.3	22	11	5.2	138	18	12.2	19.5	orinase (30 U)

* At time serums were drawn. Some of the insulin-resistant subjects had previously required larger doses.
† All values times power of 10 at head of column.
‡ Determined from 1 : 10 dilution of serum.
§ 4,800 units in 48 hours. Serum drawn after first 1,000 units administered.
‖ Approximately 20,000 units in week prior to day serum was drawn.
¶ 14,000 units day of admission to hospital 10 days previously; irregular dosage for next week; 2,700 units on day serum was drawn.
** All diabetic except Pr. who is a nondiabetic schizophrenic patient.
†† Indicates previous insulin dosage. Insulin therapy had been discontinued 14 days (D. C.) to 1 yr. (A. R.) previously.

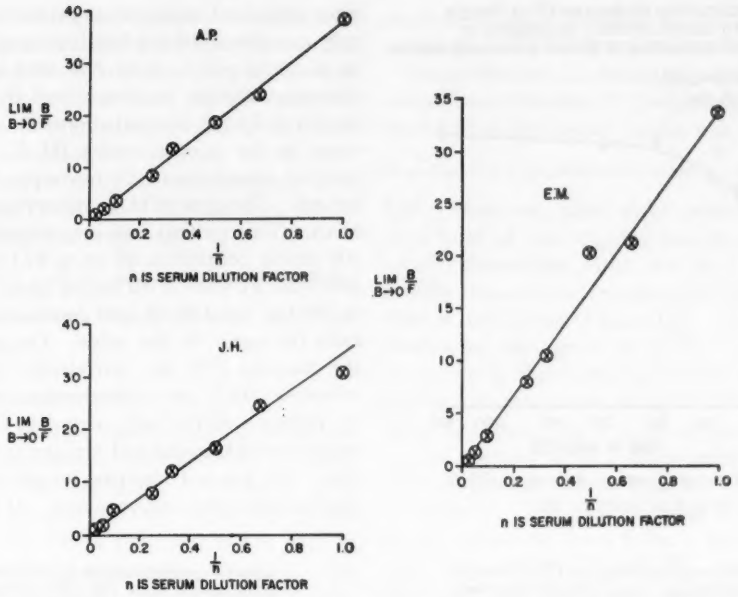


FIG. 7. $\frac{B}{F}$ VS. $\frac{1}{N}$ IN THREE ANTISERUMS

Serum in J. H. taken at a different time than the serum for which data are reported in Table I and Figures 10 and 11. Refer to Figure 1 for departure from linearity expected for different $\frac{K_2}{K}$ values on the assumption that insulin is divalent.

good fit was always possible for the case of a univalent insulin reacting with two orders of antibody combining-sites (Table I, Figure 6). Calculations from the outlined method of curve-fitting (see Appendices A and B) based on a univalent insulin reacting with two orders of antibody sites gave values for K_a and K_b of the order of 10^8 to 10^{10} and 10^7 to 10^8 L. per Mole, respectively (Table I). In many cases, if calculated for a divalent insulin, K would not have been less than one-third to one-half of the higher of the K value ranges given above.

Although exceptions are evident, the equilibrium constants were generally considerably higher in serums from nonresistant subjects than from resistant patients.

The determination of total available combining-sites is not dependent on the model used for analysis, since it is derived simply from the maximum amount of insulin bound at saturating concentrations of insulin (see Appendix B). Serums of high binding capacities may be diluted appreciably for these determinations. The values obtained at different serum dilutions were generally in excellent agreement. Where antibody concentrations are low, undiluted serum may be used. On the assumption that one molecule of 6,000 molecular weight insulin is bound by one molecule of 160,000 molecular weight antibody, less than 1 mμg. absolute amount of antibody (in 0.1 ml. plasma) is detectable by those methods provided a high specific activity insulin-I^{131} is employed. If the weight ratio, insulin/antibody, is greater than 6,000/160,000, the amount of detectable antibody is still lower. The technique employed here may well be the most sensitive method available for antibody detection yet described.

It is evident (Table I) that much greater quantities of insulin are bound in insulin-resistant patients than in serums from nonresistant patients. In addition to the cases presented in Table I, we have previously reported (1) on antiserums from 28 nonresistant insulin-treated subjects with maximum binding capacities not exceeding approximately 10 units per L. and have

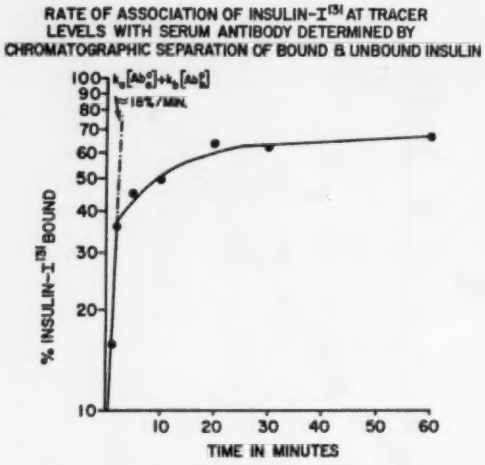
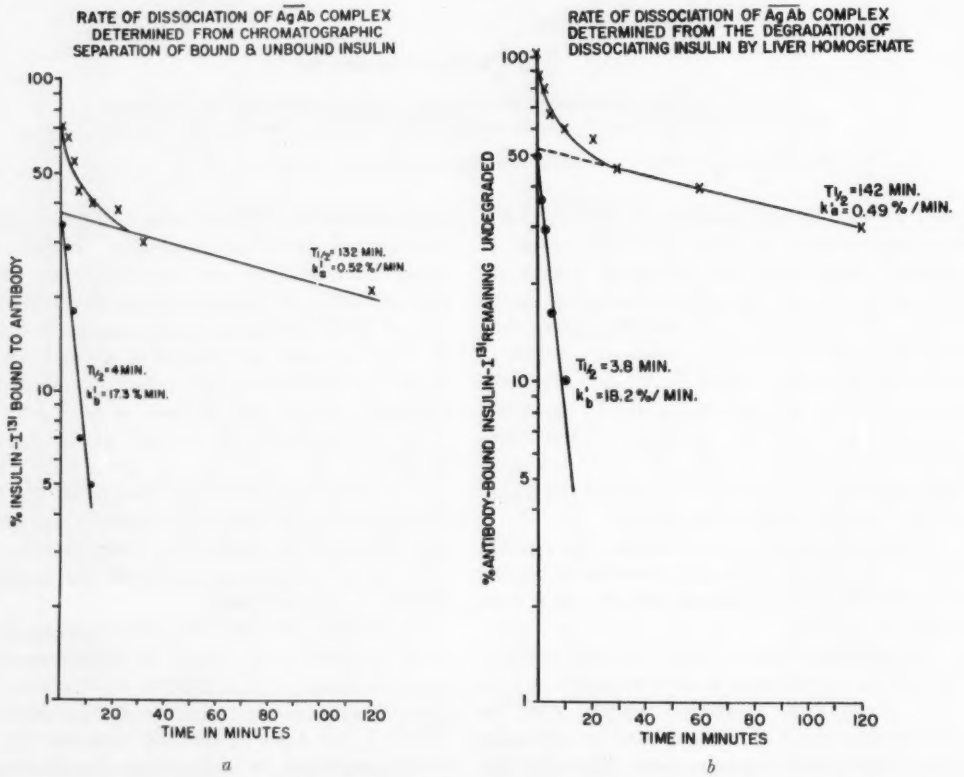

RATE OF ASSOCIATION OF INSULIN-I[131] AT TRACER LEVELS WITH SERUM ANTIBODY DETERMINED BY CHROMATOGRAPHIC SEPARATION OF BOUND & UNBOUND INSULIN
% INSULIN-I[131] BOUND
TIME IN MINUTES

FIG. 8. RATE OF BINDING OF INSULIN-I[131] IN SERUM OF SUBJECT RU.

since examined many other similar cases. Only very occasionally have binding capacities as high as 20 units per L. been observed in serums of diabetic subjects requiring less than 75 units insulin daily for adequate control. In two such cases in the present series (H. L. and L. Sy.) binding capacities of 22 units per L. were observed. The case of H. L. deserves special comment. This patient had maintained exceptionally stable control on 55 units PZI daily for the previous two years with fasting blood sugar levels of 200 mg. per 100 ml. and occasional 1+ or 2+ tests for sugar in the urine. On admission to the hospital PZI was withdrawn immediately, following which the patient maintained precisely his previous status with regard to fasting blood sugar concentrations and urinary spillage of glucose. He has not required insulin since its discontinuance over one year ago. It is of further


RATE OF DISSOCIATION OF Ag Ab COMPLEX DETERMINED FROM CHROMATOGRAPHIC SEPARATION OF BOUND & UNBOUND INSULIN
% INSULIN-I[131] BOUND TO ANTIBODY
TIME IN MINUTES
RATE OF DISSOCIATION OF Ag Ab COMPLEX DETERMINED FROM THE DEGRADATION OF DISSOCIATING INSULIN BY LIVER HOMOGENATE
% ANTIBODY-BOUND INSULIN-I[131] REMAINING UNDEGRADED
TIME IN MINUTES

a *b*

FIG. 9. RATE OF DISSOCIATION OF INSULIN-I[131]-ANTIBODY COMPLEXES IN SERUM OF SUBJECT RU.: *a*) DETERMINED BY PAPER CHROMATOGRAPHY; *b*) DETERMINED FROM RATE OF DEGRADATION BY LIVER INSULINASE

interest that his antibody concentrations showed no significant decline over a period of six months. It seems reasonable to conclude that this patient was deriving little effect from the administered PZI and that if his endogenous insulin secretion had been inadequate to maintain satisfactory control, his exogenous insulin requirements might well have been considerably greater than the 55 units per day he was receiving.

Plots of $\frac{B}{F}$ vs. $\frac{1}{n}$ revealed only a linear relationship in all antiserums tested, three of which are shown in Figure 7.

Transient state experiments

In all cases studied there appeared to be two grossly different rates at which insulin at tracer concentrations became bound to antibody (Figure 8); *i.e.*, on analysis, the approach to the equilibrium value took the form of the sum of two exponential components. Similarly, the formed antigen-antibody complexes were heterogeneous in that two rates of dissociation were observed (Figures 9a and b). The more rapid of these rates ranged from about 3 per cent per minute to 25 per cent per minute and the slow rates ranged from less than 0.01 per cent per minute to 1 per cent per minute in different antiserums (Table I). In one serum, the rates of dissociation were studied both with liver insulinase and by paper chromatography; the values for each of the two rates were in reasonable agreement by the two methods (Figures 9a and b).

In the last case and in most of the others, the observed rate of formation of complexes ($k_a[Ab^o_a] + k_b[Ab^o_b]$) at zero time agreed reasonably well with the sum of the expected values calculated from each observed reverse velocity constant and $\frac{B}{F}$ ratio according to the formula: $\lim_{B \to 0} \frac{B}{F} = K[Ab^o] = \frac{k[Ab^o]}{k'}$ (Table I). The agreement in these cases is all the more gratifying since the values for the $K[Ab^o]$'s were obtained from equilibrium studies whereas the k''s were obtained from transient state experiments. However, in several experiments (A. R., D. C., L. Sy., Table I) the observed rate of complex formation was significantly greater than the calculated rate. It could not be determined whether these discrepancies were attributable to experimental errors or to defects in the theoretical models.

At equilibrium, in experiments with tracer concentrations of insulin-I^{131}, frequently half or more than half of the bound insulin was in the slowly dissociating complex even when $\frac{B}{F}$ was as low as 0.65. However, after short periods of incubation, most of the complex was generally in the rapidly dissociable form, the ratio of slowly to rapidly dissociating components increasing with time of incubation (Figure 10). Transient state studies on the serum of J. Ha. were analyzed according to the models of univalent insulin reacting with two orders of antibody sites (Equations 32 through 34, Figure 10) and of divalent insulin reacting with a single order of antibody sites (Equations 38 through 40; curve analysis not shown). The reaction rates of the two complexes calculated from F vs. t for both models were quite similar[12] (Table II). Unfortunately, therefore, this experiment provided no grounds for choice between univalent and divalent insulin models. In both cases the calculated reverse velocity constant of the slowly dissociating component (k'_a or k'_e, respectively, in each of the two models) was greater than that observed, whereas the other calculated rate constants agreed quite well with directly observed values. The values for $K_a[Ab^o_a]$ and $K_b[Ab^o_b]$ obtained in the transient state studies according to the univalent insulin model were in good agreement with those derived from the equilibrium state analysis (Figure 11, Table III).

In all cases the initial segments of "dissociation" curves showed the sharpest slopes as in Figures 9 and 10.

Relative amounts of rapidly and slowly dissociating complexes as a function of antibody concentration

It has been noted that if the two different complexes result from the combination of univalent insulin with each of two different antibody combining-sites, the ratio between the two complexes is not affected by dilution of the antiserum; thus, although the ratio of $\lim_{B \to 0} \frac{B}{F}$ is lowered proportionately to the extent of dilution, the ratio

[12] This is to be expected as a formal consequence of the mathematics of the two systems when the reaction rates of the two complexes differ markedly.

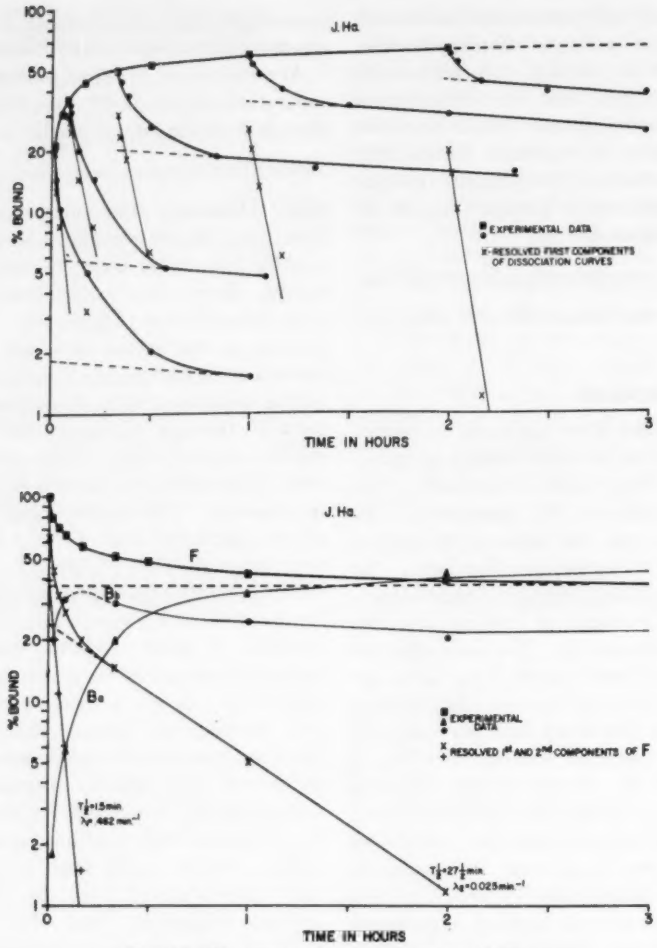


FIG. 10. TRANSIENT STATE ANALYSIS IN J. HA.

The curve joining the solid squares in the upper half of the figure represents the rate of binding of tracer insulin-I^{131}. At 0.5, 5, 20, 60 and 120 minutes, aliquots were removed to tubes containing swamping amounts of unlabeled insulin. The curves joining the solid circles represent the rates of dissociation of the insulin-I^{131} bound at these times. The latter curves were resolved into two components by extrapolating the terminal segments to the times marking onset of "dissociation." The ordinate values of the latter extrapolations are plotted as solid triangles in the lower half of the figure and represent the slowly dissociating component (B_a) according to the univalent insulin model. The values for B_b are obtained by subtracting the values for B_a from the values for total bound insulin-I^{131}.

between rapidly and slowly dissociating forms should not be altered. If, on the other hand, insulin is divalent, the ratio between the two complexes, $\frac{[\overline{AgAb_2}]}{[\overline{AgAb}]}$, will be expected to decrease proportionately with the dilution factor (cf. Equation 19). Since the experimental observations indicate that the more rapidly formed complex dissociates more rapidly and that, with time, the slowly dissociating complex increases

partly at the expense of the rapidly dissociating complex, the divalent insulin model would require that $\overline{AgAb}$ be the rapidly formed and rapidly dissociating complex and that $\overline{AgAb_2}$ be the slowly dissociating complex. This follows also from the observation that the dissociation curves show the sharpest slopes initially. The ratio between slowly and rapidly dissociating complexes should then decrease proportionately with the dilution factor. To test the applicability of each of these models to the insulin-antibody reaction, insulin in tracer concentrations was incubated with antiserum at four- to tenfold differences in dilution, and dissociation of the complexes in the equilibrium mixtures was studied by the methods described. The ratio between rapidly and slowly dissociating components and the rates of dissociation were essentially the same in different dilutions of the same antiserum even when the mixtures were preincubated for as long as 20 hours (Figure 12). The initial slopes of the dissociation curves were always steeper than the final slopes, which in itself would require, in the case of divalency of insulin, that k'_e be less than k'.

As expected, the ratio between slowly and rapidly dissociating components is lower at load insulin concentration than at tracer insulin concentration (Figure 12). This was to be expected with either of the postulated models.

TABLE II

*Rates of association and dissociation of complexes calculated for univalent insulin reacting with two orders of antibody sites (mammillary system) and for divalent insulin reacting with a single order of antibody sites (catenary system)**

	1 → 10 Dilution of J. Ha. serum Mammillary system (univalent insulin)	
	Observed from association and dissociation curves of B_a vs. t, B_b vs. t	Calculated from F vs. t, $B_{b_{eq}}$ and $B_{a_{eq}}$
k'_a	0.0021 min.$^{-1}$	0.015 min.$^{-1}$
k'_b	0.173–0.346 (mean 0.23)	0.285
$k_a[Ab^\circ_a]$	0.015	0.0170
$k_b[Ab^\circ_b]$	0.160	0.170
	Catenary system (divalent insulin)	
	Observed and calculated from dissociation data†	Calculated from F vs. t, a_{eq} and b_{eq}
k'_e	0.0021 min.$^{-1}$ (observed)	0.0092 min.$^{-1}$
k'	0.23 (observed)	0.290
$k_c[Ab^\circ]$	0.0040 (calculated)	0.0177
$k[Ab^\circ]$	0.130 (calculated)	0.170

* The calculated values from F vs. t for both models are to be compared with each other and with the observed values in the upper half of the table. Since the values for $k[Ab^\circ]$ and $k_c[Ab^\circ]$ calculated from the observed dissociation data reflect errors in the latter, they are not directly comparable with other values.

† Calculations from dissociation data: $\bar{a}$ and $\bar{b}$ vs. t were calculated from Equations 46 and 47 and extrapolated to yield a_{eq} and b_{eq}; $k[Ab^\circ]$ and $k_c[Ab^\circ]$ were then calculated from Equations 38 and 39, respectively, for equilibrium conditions $\left(i.e., \frac{dF}{dt} = 0; \frac{d[\overline{AgAb}]}{dt} = 0\right)$ employing the mean values of k' and k'_e observed from the dissociation curves.

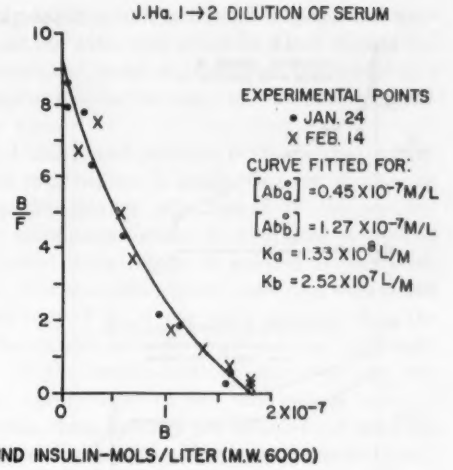


FIG. 11. $\frac{B}{F}$ vs. B IN SERUM OF SUBJECT J. HA.

DISCUSSION

The antigen-antibody system under study here is unusual in several respects. Nonprecipitability of the complexes was observed previously in antiserum obtained from nonresistant subjects (1) and had been attributed either to the extremely low concentrations of complexes or, alternatively, to the possibility that the insulin-

TABLE III

Comparison of equilibrium and transient state kinetic data for model of univalent insulin and two orders of antibody binding sites in J. Ha.

	Dilution of serum		Corrected for undiluted serum	
	1 → 2 Equilibrium	1 → 10 Transient	Equilibrium	Transient
$K_a[Ab^\circ_a]$	6.0	1.14	12	11.4
$K_b[Ab^\circ_b]$	3.2	0.60	6.4	6.0

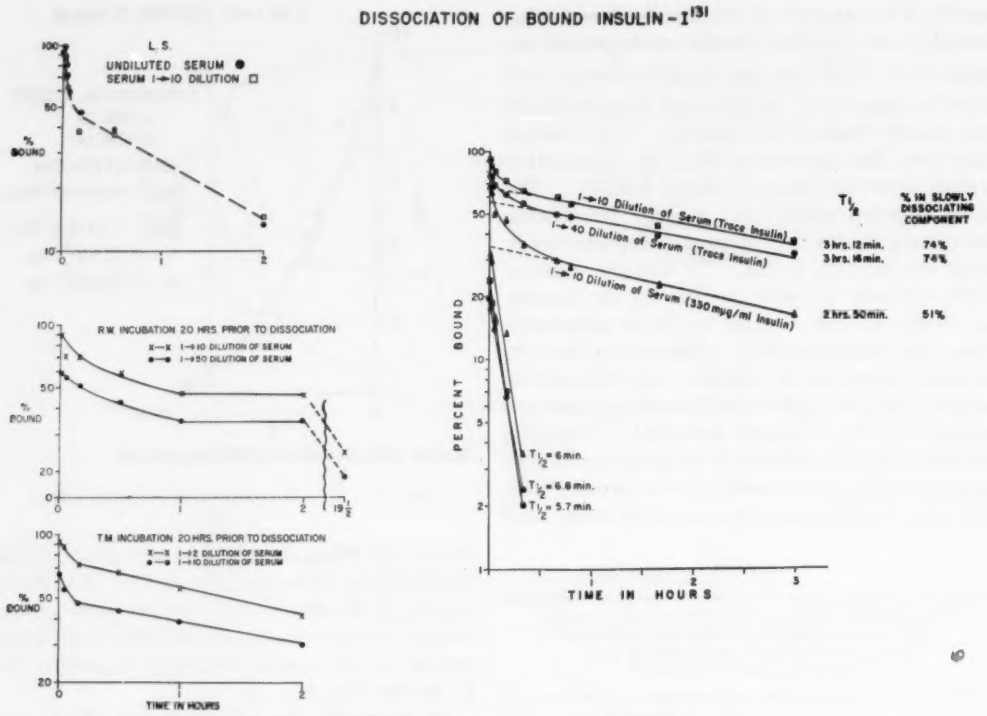


FIG. 12. "DISSOCIATION" OF INSULIN-ANTIBODY COMPLEXES FORMED AT DIFFERENT DILUTIONS OF THE SAME ANTISERUMS

Experiment on right in serum of Gr. taken at a different time than the serum for which data are reported in Table I.

binding antibody is univalent. However, in the present series, serums of high antibody titer, obtained from insulin-resistant patients, were analyzed throughout the range of marked antibody excess to marked antigen excess and precipitation could not be demonstrated even after prolonged refrigeration and centrifugation for 30 minutes at 5,000 G. Complexes containing 10 μg. insulin per ml. have been studied so that concentrations of complexes probably exceeded 100 μg. per ml. in these mixtures. With the rabbit anti-human serum albumin system it is possible to demonstrate, by paper chromato-electrophoresis (6), precipitability when complexes are present in a concentration of 1 μg. per ml. The non-precipitability of insulin-antibody complexes is therefore not due to inadequate concentrations in the mixtures employed. Although the alternative hypothesis previously offered, that insulin-binding antibody is univalent, may still be valid, univalency of antigen would suffice, equally well, to explain nonprecipitability. However, since most protein antigens are demonstrably multivalent or can be considered likely to be so on the basis of their structure even in the absence of experimental proof, the univalency of insulin as an antigen merits scrutinizing consideration.

Several different types of experiments have been performed to test whether insulin is univalent or multivalent. The results of some of these studies militate strongly against multivalency whereas the results of other experiments make multivalency appear unlikely. The evidence may be summarized.

1) On dilution of antiserum, the ratio B/F at tracer insulin concentrations falls linearly with the reciprocal of the dilution factor. If insulin were multivalent, B/F should be a function of some power of the dilution factor.

2) The ratio between rapidly and slowly dissociating complexes formed at *trace* insulin concentrations is not affected by four- to tenfold

dilution of antibody concentration in individual antiserums. It has been pointed out that if "multivalent complexes" existed, they should be more prevalent at higher than at lower antibody concentrations and that the more dilute antiserum should contain a smaller proportion of slowly dissociating complexes. Furthermore, antiserum of very low antibody concentration should be virtually free of slowly dissociating complexes if insulin is multivalent. These requirements are incompatible with the experimental observations.

For reasons given below, the possibility of an insulin valency greater than two may be excluded almost with certainty. If, therefore, consideration is restricted to the possibility of divalency as opposed to univalency, the following points argue against divalency.

3) In all antiserums, the two single order rate constants of dissociation (for release of insulin) differ by a factor of 50 to 100. As emphasized above, were the rapidly and slowly dissociating complexes to be correlated with "univalent complexes" and "divalent complexes," the latter would have to be identified with the slowly dissociating complexes. It then becomes difficult to explain why the rate of release of insulin from "divalent complexes" should be *so much slower* than from "univalent complexes" without postulating that the presence of a second antibody molecule increases, many fold, the strength of binding of insulin in the complex (*i.e.*, $k'_c \ll k'$). Although such a possibility is not out of the question, especially in view of the suggestion of Talmage (7) that inter-antibody linkages may be formed, it appears unlikely to us in the present instance.

4) The observation that most of the bound insulin may be in the slowly dissociating component even when as little as 40 per cent of trace concentrations of insulin is bound to antibody is incompatible with the slowly dissociating component being "divalent complex" unless K_c is significantly greater than K.

5) The experimental curves for B/F vs. B in the equilibrium studies could frequently not be fitted to theoretical curves derived from the model for a divalent insulin and a single order of antibody combining-sites. In these cases, the experimental curves were actually of a form completely incompatible with such a model but were compatible with, and could be fitted closely by, a theoretical curve derived from the model of a univalent insulin reacting with two different antibody sites.

6) Finally, independent evidence that insulin is not multivalent is available from studies on the sedimentation velocities of I^{131}-insulin-antibody complexes formed in undiluted serums of high titer in the region of marked antibody excess. These sedimentation velocities were found to be only 5 to 6 per cent greater than the sedimentation velocity of I^{131}-labeled γ-globulin (8). If the insulin-binding antibody does not differ significantly in size and shape from γ-globulin, these findings are inconsistent with the presence of complexes that contain more than a single antibody molecule regardless of the geometrical configuration of the complex, and are certainly incompatible with complexes containing more than two antibody molecules.

Thus, heterogeneity of antibody combining-sites rather than multivalency of insulin is favored as an explanation for the heterogeneity of insulin-antibody complexes. The studies of Karush (9) have revealed that antibodies produced against haptenic dye groups react with the haptens in a manner consistent with the conclusion of Pauling, Pressman and Grossberg (10) that the distribution of free energy of binding can be described by a Gaussian error function. Therefore, even a single "order" of antibody combining-sites may be expected to react heterogeneously to some degree. However, the two widely different rates of dissociation of complexes in the insulin-antibody system cannot readily be interpreted simply in terms of a normal distribution of binding energies, but must be ascribed to groups of distinctly different antibodies. That a distribution of energies within each group may exist can hardly be denied. The K values being measured simply represent average values within each group.

The evidence cited for the presence of two orders (at least) of antibody combining-sites has been indirect, derived essentially from exclusion of alternative models and from consistency of the experimental data with a theoretical model. However, in studies reported elsewhere (11) it has been shown that crystalline insulins obtained

from different mammalian species show marked differences in direct reaction and in competitive cross-reactions with individual antiserums. Thus, beef and sheep insulins usually bind much more strongly than does pork or horse insulin and at certain concentrations the presence of beef insulin or sheep insulin produces, by competitive inhibition, a lower B/F ratio for pork insulin-I^{131} and horse insulin-I^{131} than do pork insulin and horse insulin themselves at the same concentrations. When pork or horse insulin is added in concentrations sufficient to result in a very low B/F ratio for these insulins, beef insulin-I^{131} may still be bound at a relatively high ratio. Yet, at low insulin concentrations, pork insulin is at least as effective as beef insulin in inhibiting the binding of pork insulin-I^{131}. These observations are incompatible with a single order of antibody combining-sites but are consonant with the presence of more than one order of antibody combining-sites, each having different affinities for each species-specific insulin. Inasmuch as commercial insulin preparations are composed of mixtures of pork and beef insulins, it is tempting to attribute the heterogeneity of antibody combining-sites to the different antigenic determinants present in beef and pork insulin. However, there is no experimental evidence on which to base such a correlation and, in fact, there is evidence that several different antibodies may be formed even in response to a single, relatively small, antigenic site. Landsteiner and van der Scheer (12) established the formation of discrete antibodies directed toward closely adjoining determinant groups and Kabat (13) has demonstrated heterogeneity of antibodies even to the simple repeating α 1, 6 glucose links of dextran, the antibody combining-sites varying in size from that complementary to a trisaccharide to that complementary to a straight chain hexasaccharide. Harris, Sanger and Naughton have shown that insulins derived from five different mammalian species differ only in positions 8 to 10 of the glycyl (A) chain (14). The sequence in these positions in beef insulin is ala, ser, val; in sheep insulin ala, gly, val; in pork insulin thr, ser, ileu and in horse insulin thr, gly, ileu. The differences in reaction with all these insulins which are identical except at the site under discussion, at least so far as amino acid sequence is concerned, suggest strongly that this site is an antigenic site. But antigenic determinacy could reside in several groups in this site and antibody molecules directed to several of these groups could differ strikingly in their affinity for insulin. It is therefore not possible to assign the different antibody combining-sites to beef insulin antibody and to pork insulin antibody specifically. Studies with serums from persons immunized with a single species insulin are obviously indicated. However, it is of some interest that rabbit anti-beef insulin serum[13] forms heterogeneous complexes with beef insulin.

It should be emphasized here that evidence favoring univalency of insulin in its reaction with antibody does not necessarily imply that there is but a single antigenic site. The presence of two or more antigenic sites in insulin is quite compatible with univalency of insulin inasmuch as steric restrictions might well account for the inability of a single relatively small insulin molecule to bind antibody at more than a single antigenic site even if multiple sites are present.

It has been noted that the K's were generally lower in serums with higher antibody concentrations. Although there are definite exceptions, the general tendency would suggest that in this system, at least, a marked increase in antibody synthesis is associated with the production of antibody of lower affinity for the antigen.

Several other matters remain for critical comment. It has already been observed that if the antibody molecules are assumed to be divalent, the possibility cannot be excluded that the equilibrium constant for each site might be altered by the presence of an insulin molecule at the other site. It has not been feasible to evaluate this possibility, which is of importance only toward the side of antigen excess, nor are we aware of any relevant studies in any other immunologic system. It is clear that this complication may be ignored in the region of marked antibody excess and can in no way be considered responsible for the heterogeneity of complexes at trace insulin concentrations.

Another possible complication resides in the characteristic tendency of insulin to polymerize at high concentrations. In this work insulin has been assumed to be homogeneous and calcula-

[13] We are indebted to Drs. E. Arquilla and A. Stavitsky for this antiserum.

tions have been based on a molecular weight of 6,000. Whether the individual insulin molecule weighs 6,000, 12,000 or 24,000 is of little importance in the results since a constant factor of 1, one-half or one-fourth need only be applied to the calculated molar concentrations. However, should monomers, dimers, trimers, and so forth coexist in the mixtures being studied, analysis of the kinetics of reaction with antibody would become extremely complicated, for not only might a single antibody combining-site be complexed with one, two, three or more insulin monomer units but also, in the process of formation of dimers, trimers, and so forth, the antigenic sites might be masked or shielded in such a way as to inhibit or prevent reaction with the antibody. Conversely, if insulin polymerized into such a form as to leave the antigenic sites of several of the monomers exposed, it might be possible to form large antigen-antibody aggregates despite univalency of the insulin monomer. It has already been noted that ultracentrifugal sedimentation studies exclude the last possibility. Moreover, it appears unlikely that significant amounts of insulin polymers were ever present under the conditions of the experiments reported here. The studies of Fredericq (15) have provided evidence that, in concentrations < 0.1 per cent, the 6,000 molecular weight monomer predominates above pH 9 with preparations dialyzed free of zinc.[14] In the present study the solutions employed never exceeded 0.001 per cent and usually were in the range 0.000001 to 0.00005 per cent. It seems highly improbable that the percentage of dimers in these solutions could ever have been significant.

Finally, a word may be said about the thermodynamics of the reaction of insulin with insulin-binding antibody. The standard free energy change (ΔF°), calculated from the equilibrium data according to the reaction isotherm,

$$\Delta F^\circ = - RT \ln K$$

amounts to about minus 10 to 11 Kcal. per Mole and minus 11 to 14 Kcal. per Mole for reaction with each of the two antibody combining-sites, respectively (see Appendix B). These values for the free energy of binding are considerably greater than those reported by Karush (9) for the azohaptenic dye-antibody systems (about 7 Kcal. per Mole) or for other protein-antiprotein systems (16). As indicated earlier, even if the equilibrium constant were calculated on the basis of a divalent insulin model, $-\Delta F^\circ$ would still be in the range of 12 to 13 Kcal. per Mole in many cases.

TABLE IV

Thermodynamic constants in Subject M. S.

Antibody sites	$\Delta F^\circ_{310^\circ K.}$	$\Delta H^\circ_{294^\circ K.}$	ΔS°
	Kcal./Mole	*Kcal./Mole*	*cal./° K./Mole*
"a"	−12.9	−3.6	30.0
"b"	−11.8	−3.6	26.5

Some preliminary studies on activation energy and on enthalpy changes have been made. It has been observed that both equilibrium constants are increased approximately twofold when the temperature of the reaction is lowered to 4° C. and that at this temperature the reverse velocity constant is decreased by a factor of about 10. Because of the presence of two components in the complexes and the long periods of time required to complete observations at lower temperatures, the data necessary for reliable calculations are difficult to obtain. In one case followed for 72 hours, the equilibrium constants were just twice as great at 4° C. as at 37° C. The calculated values for ΔF°, ΔH° and ΔS° are given in Table IV. In view of the very high binding energy in this antigen-antibody system, these studies should be of interest and are being pursued further.

A detailed discussion of the clinical significance of insulin-binding antibodies is not necessary here. However, it should be emphasized that a variety of factors must be taken into consideration before attempting to make a correlation between insulin requirements and insulin-binding capacities in individual cases. Among subjects with low binding capacities (< 10 units per L. plasma) there is little demonstrable relationship between insulin dosage and antibody-binding activity simply because the latter is too small to be responsible for significant insulin wastage and

[14] In the present studies duplicate runs in the usual veronal buffer, pH 8.6, and in glycine buffer, pH 10, have yielded identical results for binding of insulin to antibody. All insulin-I^{131} solutions were thoroughly dialyzed and it has already been noted that insulin-I^{131} showed no differences from unlabeled insulin with respect to binding by antibody.

because many of these patients do retain some ability to secrete endogenous insulin so that insulin dosage does not reflect insulin requirements accurately. However, it seems fairly certain that high binding capacities (> 50 units insulin per L. plasma) are likely to be accompanied by some degree of insulin resistance at least, and that the very highest binding capacities are associated with the most patent evidence of clinical resistance.

The question that must be asked is—How does the presence of insulin-binding antibodies result in an increase in insulin requirements? It might appear that antibody-induced insulin resistance should be no more than a transient phase if a steady state is eventually reached between association and dissociation of the antigen-antibody complexes since, even in the presence of high antibody concentrations, saturation of the antibody would eventually occur on continued insulin therapy. The transient nature of such insulin resistance may be noted on occasion when acute increases in insulin requirements occur, especially during infections, and when after a short period of massive insulin therapy there follows a period of repeated insulin reactions for days after discontinuation of insulin. The latter phenomenon has been interpreted (17) as due to gradual release of insulin from dissociating insulin-antibody complexes following the initial accumulation of high concentrations of insulin in these complexes. Nevertheless when such patients continue to require large doses of insulin for many months or years, it must be concluded that much of the insulin is being destroyed without the benefit of hormonal action. It seems reasonable to presume that such insulin wastage is attributable to the immunologic elimination of circulating antigen-antibody complexes and subsequent proteolytic destruction of the contained insulin moiety. Assuming a 6 L. apparent volume of distribution for antibody, an insulin binding capacity of 200 units per L. plasma would mean a total insulin-binding capacity of 1,200 units. If, on an insulin dosage schedule required to maintain adequate control of diabetes, the antibody was on the average half saturated and if the insulin antibody complexes were eliminated from the circulation with a half-time of two days,[15] insulin requirements would be 210 units per day above those necessary for hormonal activity.

It is therefore evident that many factors other than antibody concentration *per se* will enter into determining the effect of antibodies on insulin requirements in individual cases. Among these may be listed: *a*) the rate at which insulin-antibody complexes are removed from the circulation which together with the rate of turnover of uncomplexed antibody will equal the rate of antibody synthesis in the steady state; *b*) the ratio of bound to free insulin at the free insulin concentration necessary for control of diabetes; *c*) the extent of saturation of the antibody at this ratio; and *d*) the rate at which complexes form and dissociate (which will determine how much of a given insulin dose will escape to the tissues before being bound or rebound to antibody). There may still remain many other factors not yet considered, but it is hoped that future study will aid in their elucidation.

[15] This is the case with soluble complexes of the rabbit anti-bovine serum albumin and γ-globulin systems (18).

SUMMARY AND CONCLUSIONS

1. Kinetic aspects of the reaction between insulin and insulin-binding antibody were studied with antiserums from insulin-resistant and nonresistant human subjects.

2. Insulin-antibody complexes are soluble throughout the range of marked antibody excess to marked antigen excess.

3. In antiserums from nonresistant subjects, insulin-binding capacities generally did not exceed about 10 units insulin per L. serum, whereas insulin-binding capacities ranged from 50 units per L. to over 500 units per L. in antiserums from insulin-resistant patients.

4. The methods employed permit detection of less than 1 mμg. insulin-binding antibody, assuming the molecular weight of insulin to be 6,000.

5. Methods are presented for the analysis and evaluation of equilibrium state and transient state data in systems composed of univalent or multivalent antigens reacting with a single order or with multiple orders of antibody combining-sites. Where necessary, antiserums were diluted appreciably for the purpose of slowing the rates of reaction sufficiently to permit evaluation of the rates of formation of complexes.

6. Heterogeneity of insulin-antibody complexes is revealed by both equilibrium state and tran-

sient state studies. It appears, from the results of a variety of experimental approaches, that insulin is univalent in its reaction with antibody and that there are (at least) two orders of antibody combining-sites ("a" and "b") present in most antiserums, the "a" term being given to the sites with the greater affinity for insulin. Univalency of insulin is not necessarily to be equated with mono-antigenicity inasmuch as binding of more than a single antibody molecule at different possible antigenic sites is conceivably prohibited by steric hindrance.

7. The ranges of values for the equilibrium constants, K, the forward velocity constants, k, and the reverse velocity constants, k′, where formation of the complex is taken as the forward direction, are as follows:

$K_a = 1.3 - 180 \times 10^8$ L. per Mole,
$K_b = 1.0 - 46 \times 10^7$ L. per Mole;
$k_a = 2 - 145 \times 10^5$ L. per Mole per minute,
$k_b = 6 - 510 \times 10^5$ L. per Mole per minute;
$k'_a = 4 - 93 \times 10^{-4}$ minute^{-1},
$k'_b = 3.4 - 35 \times 10^{-2}$ minute^{-1}.

8. The equilibrium constants were generally significantly higher in serums of nonresistant subjects (low antibody concentrations) than in serums of resistant subjects (high antibody concentrations). These findings imply that an increased rate of synthesis of insulin-binding antibody may be associated with the production of antibody of lower affinity for the antigen.

9. The standard free energy changes for the reactions with "a" and "b" antibody sites are in the range, minus 11 to 14 Kcal. per Mole and minus 10 to 11 Kcal. per Mole, respectively. Preliminary data indicate a change in heat content of about -3.5 Kcal. per Mole and a positive entropy change of 25 to 30 entropy units in the formation of insulin-antibody complexes.

10. The relationship of the results of these studies to certain aspects of clinical insulin resistance has been considered briefly.

APPENDIX A

Method of curve-fitting. A number of methods may be employed but the following has been found most expedient. Asymptotic straight line slopes are drawn to the curves at their extremities near the x and y axes. These slopes approximate K_b and K_a, respectively. The sum of the ordinate intercepts of the two straight lines is then adjusted to equal the ordinate intercept of the experimental curve by "moving" both straight lines to the left keeping their slopes constant. The intercepts on the x axis will now approximate the respective [Ab°]'s.

Using these values for the K's and [Ab°]'s, B/F ratios for different values of F are then calculated for each site from Equation 5. The curve of B/F vs. B is then plotted using Equation 9 and compared with the experimental points. The K's and [Ab°$_b$]'s are now adjusted until the calculated curve makes a good fit to the experimental points. Little variation in the K's and [Ab°]'s is tolerated if the fit to the experimental points is not to be disturbed.

APPENDIX B

Notes on experimental errors

Maximum binding capacities. The presence of damaged components in insulin-I^{131} does not seriously affect the higher B/F ratios but produces an increasing error as the ratios fall significantly below 1. However, the maximum binding capacity is determined not from single mixtures at high concentration but from the best curve extrapolated from values at lower concentrations (and therefore higher percentages bound) to the x axis. Thus, while individual calculations at the highest concentrations might vary by as much as 50 per cent in the quantity of bound insulin, the maximum binding capacities determined by the best extrapolations are estimated to be in error by not more than 10 to 15 per cent as judged from the reproducibility of replicate determinations.

Concentrations of antibody combining-sites. These are determined from the fitted curves as described in Appendix A and depend in some degree on the accuracy of all the points. However, [Ab°$_b$] generally comprised 80 per cent or more of the total antibody combining-sites and the error in this value is therefore of the same order as that in the maximum binding capacity. The value for [Ab°$_a$] is dependent on the initial slope of the B/F vs. B curve. [Ab°$_a$] (comprising only the smaller fraction of total antibody combining-sites) therefore is much more sensitive to experimental errors of the points close to the ordinate axis. It is estimated that the values for [Ab°$_a$] may be in error by as much as twofold.

Equilibrium constants. The accuracy of these values depends on the accuracy of the [Ab°]'s and $\lim_{B \to 0}$ B/F ratios for each site. The error is directly proportional to the error of the $\lim_{B \to 0}$ B/F ratio (which is small) and inversely proportional to the error of the [Ab°]. Accordingly, K_b is probably accurate to ± 10 to 20 per cent wherein K_a may be in error twofold reciprocally to the error of [Ab°$_a$].

Standard free energy change. A twofold error in K produces an error of about 0.3 in $\Delta F°$. Since the latter ranged from about 9 Kcal. per Mole to 14 Kcal. per Mole, the percentage error probably does not exceed 2 to 3 per cent.

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SPECIES-SPECIFICITY OF HUMAN ANTI-BEEF, PORK INSULIN SERUM

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Previous studies have revealed the presence of insulin-binding antibodies in the serums of insulin-treated human subjects. Antigen-antibody complexes in this system do not precipitate out of solution but are readily identified by paper chromato-electrophoresis employing insulin-I^{131} (1). Kinetic studies of the reaction between crystalline beef insulin and antiserum have indicated that insulin is probably univalent and that there are at least two distinct orders of antibody-combining sites (2–4). Since insulins from five different mammalian species have been found to differ in amino acid sequence only in positions 8 to 10 of the A (glycyl) chain (5), it appeared of interest to study the reactivity and cross reactivity of these various insulins in the same antiserum. Studies with human insulin, several different preparations of beef insulin, including beef desamido insulin, and the A and B chains of insulin are also reported.

MATERIALS AND METHODS

The following species-specific insulins and insulin fractions [1] were labeled with I^{131}: beef insulin (Lilly, Lot No. 535664), pork insulin (Lilly, Lot No. 499667), horse insulin, sheep insulin, beef insulin (Boots [2]), desamido insulin derived from the Boots beef insulin by countercurrent fractionation by Dr. Lyman Craig, A chain and B chain of beef insulin. All the aforementioned insulins were crystalline preparations. A crude preparation of human insulin (1.8 units per mg.)[3] was used in competitive cross reaction experiments but was not labeled. The insulins were labeled with an average of less than 0.6 iodine atom per molecule 6,000 molecular weight insulin and at specific activities of 10 to 40 mc. I^{131} per mg. insulin. The methods employed in preparation and in protection against severe radiation damage have been described previously (1, 7).

Mixtures containing trace amounts of I^{131}-labeled insulin and various concentrations of unlabeled insulin from the same or different animal species were incubated together with antiserums at 37° C. for four hours. Aliquots were then applied to paper strips for hydrodynamic flow chromatography and electrophoresis at 4° C. The conditions employed for these runs as well as the assay of radioactivity on the strips by means of an automatic strip counter have been described in detail previously (1). Chromatographic or electrophoretic separation of free insulin and insulin bound to antibody depends on the adsorption of free insulin to the paper at the site of application (1, 8) while antibody-bound insulin migrates with the inter-γ-β-globulins (9). The amounts of free insulin and bound insulin in each mixture were determined from the areas under the respective peaks of radioactivity in the two positions.

No differences in the binding of I^{131}-labeled insulin and unlabeled insulin from the same species could be detected, *i.e.*, at any particular insulin concentration, the same degree of binding of insulin-I^{131} was observed whether the insulin present was derived entirely from the preparation of I^{131}-labeled insulin or was primarily uniodinated insulin with only a tracer amount of iodoinsulin. It is assumed, therefore, that immunologic reactivity of insulin is not altered by the labeling procedure employed here. Analytic methods for determination of equilibrium constants and standard free energy changes have been described (4).

RESULTS

Data are presented in plots of the ratio of bound insulin-I^{131} to free insulin-I^{131} (B/F) as a function of the total insulin concentration present. It has been shown elsewhere (4) that, at concentrations of insulin negligible compared to the concen-

[1] We are greatly indebted to Doctors O. H. Behrens and C. W. Pettinga for supplies of the Lilly beef and pork insulins, to Dr. Hans Neurath for the horse insulin, to Dr. L. Craig for Boots beef insulin, desamido insulin, sheep insulin, and A and B chains, and to Doctors F. Tietze and J. Field for human insulin. The Lilly beef and pork insulins assayed at 27 to 29 units per mg. and the horse insulin at 25 units per mg. in the Lilly laboratories. The Boots beef insulin assayed at 21 to 23 units per mg. in the Boots laboratories but this insulin is said to be comparable in purity to that of the Lilly preparations (6). The desamido insulin showed the same hormonal potency as the parent preparation (6).

[2] Standardized sample of insulin from the Commission on Proteins, IUPAC.

[3] The concentration of human insulin in this preparation was based on the assumption that pure human insulin would contain 28 units per mg. Accordingly, 1 mg. of crude preparation was taken to represent 64.5 μg. human insulin.

tration of antibody ("trace insulin concentrations"), the ratio B/F is the sum of the products of antibody concentration and equilibrium constant for each reaction between insulin and the different antibodies which may be present. Thus, for the same antibody concentration a higher B/F value is indicative of a higher equilibrium constant (K)[4]

[4] $K = \frac{[\text{Ins-Ab complex}]}{[\text{Ins}]\ [\text{Ab}]}$, where Ab denotes antibody and the square brackets denote molar concentrations.

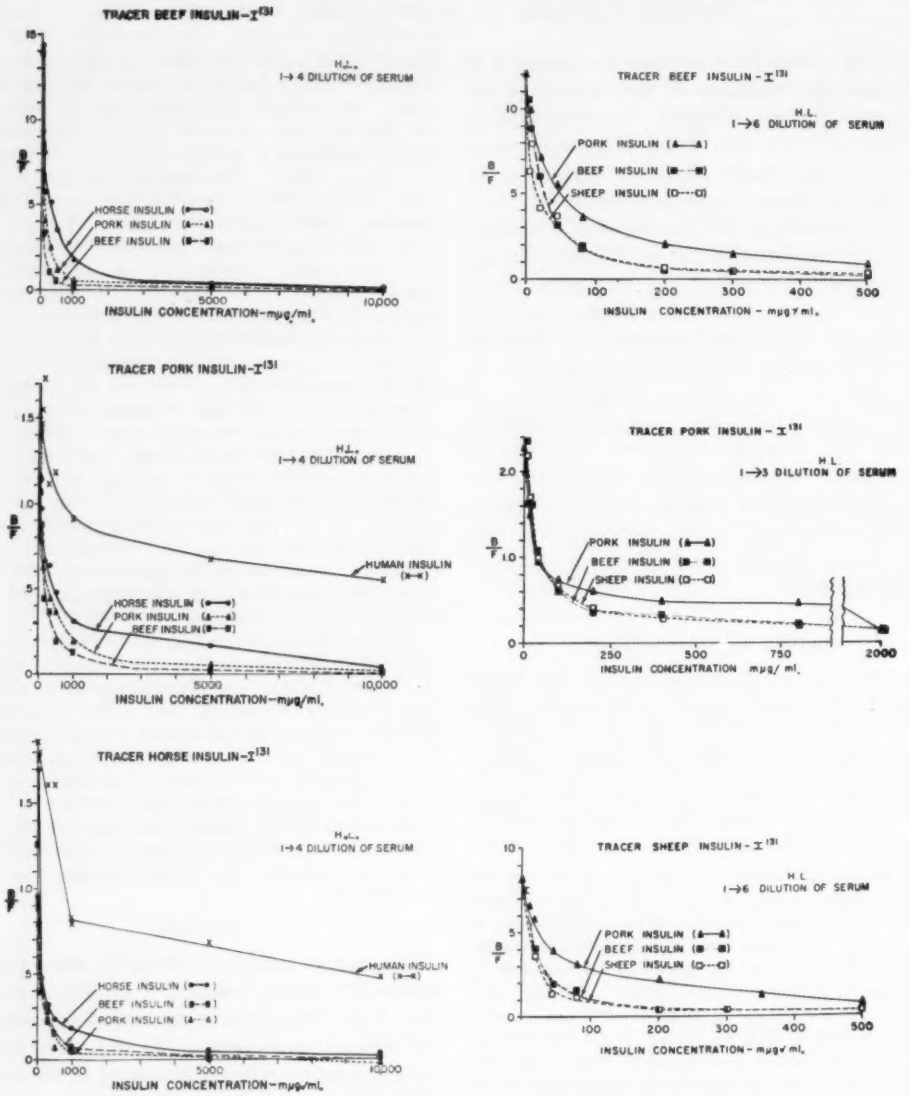

FIG. 1. SUBJECT H. L. RATIO OF BOUND INSULIN-I^{131} TO FREE INSULIN-I^{131} (B/F) AS A FUNCTION OF CONCENTRATION OF VARIOUS UNLABELED INSULINS

Human insulin in the concentrations employed failed to lower the ratio for beef insulin-I^{131} significantly at 1:4 dilution of serum but did do so at higher serum dilutions. Except where otherwise indicated, beef insulin is Lilly beef insulin.

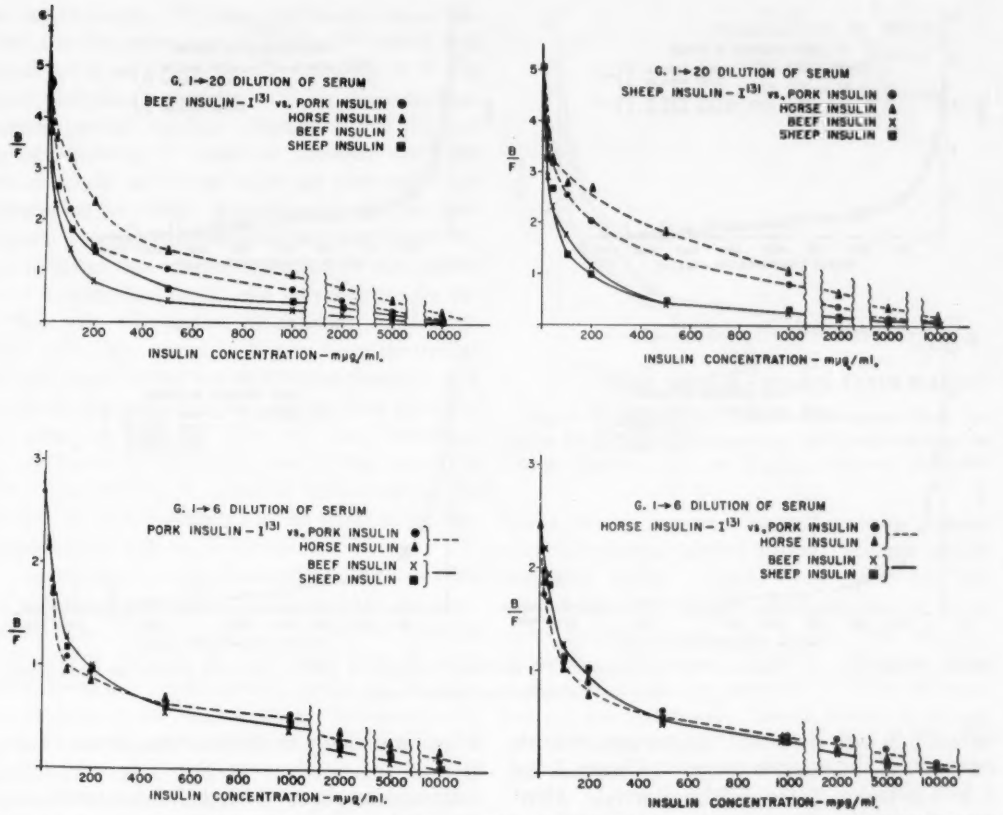

FIG. 2. SUBJECT G. RATIO OF BOUND INSULIN-I^{131} TO FREE INSULIN-I^{131} (B/F) AS A FUNCTION OF CONCENTRATION OF VARIOUS UNLABELED INSULINS

for the reaction between insulin and that antibody. It has also been shown that the B/F ratio at trace insulin concentrations falls proportionately with dilution of the serum.

In most antiserums the B/F ratio at trace concentrations of beef insulin-I^{131} is significantly greater than the B/F ratio for pork insulin-I^{131} or horse insulin-I^{131} under the same conditions. In the present series of three antiserums, which have been studied in some detail, a similar pattern was observed in two cases (H. L. and G., Figures 1 and 2) but in the third case (L. J., Figure 3) the differences among the different species-specific insulins, although definite, were small. Since the experiments in L. J. and G. were done with the same preparation of I^{131}-labeled insulin, it is clear that differences in reaction among the different species' insulins are not attributable to alterations of insulin during the labeling procedures. It should also be emphasized that experiments with the same antiserum and different preparations of the same species' insulin yield reproducible results. In H. L. and G. the binding of beef insulin-I^{131} and sheep insulin-I^{131} was much greater than the binding of horse insulin-I^{131} and pork insulin-I^{131} at trace insulin concentrations and at the same dilution of antiserum. In order to obtain convenient B/F ratios over a wide range of insulin concentrations it was therefore necessary to employ different dilutions of antiserum in the different experiments. When account is taken of the serum dilution factors it is seen that the B/F ratios at trace insulin concentrations in the absence of added unlabeled insulins were about 7 to 10 times as great for beef insulin-I^{131} and sheep insulin-I^{131} as for pork insulin-I^{131} and horse in-

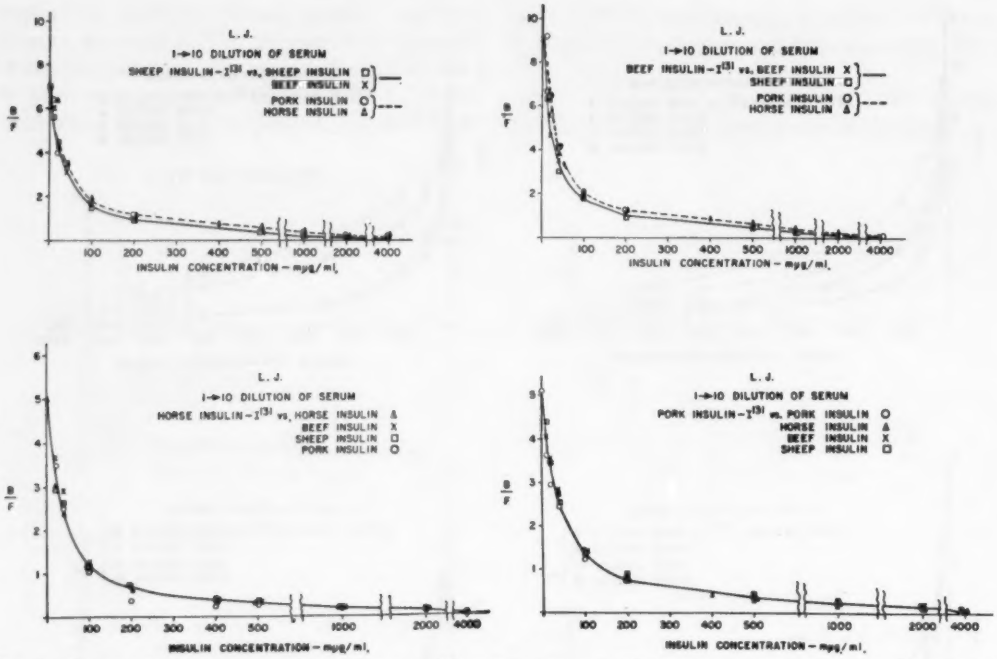

FIG. 3. SUBJECT L. J. RATIO OF BOUND INSULIN-I^{131} TO FREE INSULIN-I^{131} (B/F) AS A FUNCTION OF CONCENTRATION OF VARIOUS UNLABELED INSULINS

sulin-I^{131} in both H. L. and G. (compare ordinate intercepts of the separate frames in Figures 1 and 2 with attention to the dilution factors). However, the total binding capacities for the different insulins at high insulin concentrations did not differ significantly in the same antiserum (Table I).

In experiments in which the labeled insulin was derived from the same species as the unlabeled insulin it is possible to determine the actual amount

TABLE I

Binding characteristics of various insertions in the serum of Subject G

Insulin species	Site	Binding capacity	K	ΔF°
Beef	"a"	66×10^{-9} *Moles/L.*	1.4×10^{9} *L./Mole*	−12.8 *Kcal./Mole*
	"b"	682/748*	0.032	−10.5
Sheep	"a"	100	0.8	−12.5
	"b"	734/834*	0.027	−10.4
Pork	"a"	30	0.38	−12.0
	"b"	720/750*	0.0066	− 9.6
Horse	"a"	40	0.31	−11.9
	"b"	708/748*	0.0034	− 9.2

* Total binding capacity.

of bound insulin (B) from the insulin concentration and the percentage of insulin-I^{131} which was observed to be bound. In L. J. and G., B/F has been plotted as a function of B for the individual species-specific insulins (Figures 4 and 5). A great similarity is observed between beef and sheep insulin on the one hand and pork and horse insulin on the other. Experimental data for beef insulin reacting with human anti-beef, pork insulin serum have been shown to be compatible with a theoretical model of a univalent insulin reacting with two orders of antibody combining-sites (4). The curves in Figure 5 are theoretical curves fitted (4) for the equilibrium constants and antibody concentrations for each of these two sites as given in Table I. The "a" sites contribute primarily to the sharp upsweep of the curves in the region of low B, whereas binding of insulin to the "b" sites is reflected in the terminal shallow slopes of the B/F vs. B curves.

On competitive cross reaction, unlabeled insulins of all animal species are capable of blocking completely the binding of any of the labeled insulins (Figures 1 through 3), indicating that all sites available to any single species' insulin are likewise available to all the other insulins. However, in G. and H. L. (Figures 1 and 2) the concentration of unlabeled insulin required to reduce the binding of an I^{131}-labeled insulin to any arbitrary level depended on the species-specific insulin employed. In general, beef and sheep insulins were the strongest competitors against any of the labeled insulins, except that at low insulin concentrations pork and

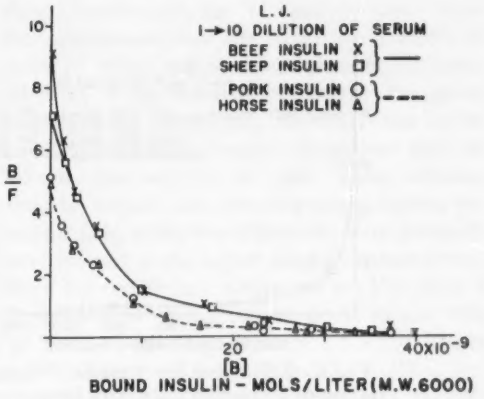

FIG. 4. SUBJECT L. J. RATIO OF BOUND TO FREE INSULIN (B/F) AS A FUNCTION OF THE CONCENTRATION OF BOUND INSULIN (B) FOR VARIOUS ANIMAL INSULINS

horse insulins competed as well or better against their homologous labeled insulin. Human insulin competed weakly (Figure 1) and was not employed in high enough concentrations in these cases to demonstrate that it can block all sites available to the animal insulins. However, with antiserums of binding capacities of 10 to 20 mμg. per ml. for beef insulin, human insulin at 10 μg. per ml. does inhibit *completely* the binding of beef insulin-I^{131}.

Lilly beef insulin, Boots beef insulin and desamido insulin derived from the latter all competed identically against Lilly beef insulin-I^{131} in G. (Figure 6). Furthermore, binding of all three labeled insulins was inhibited identically by un-

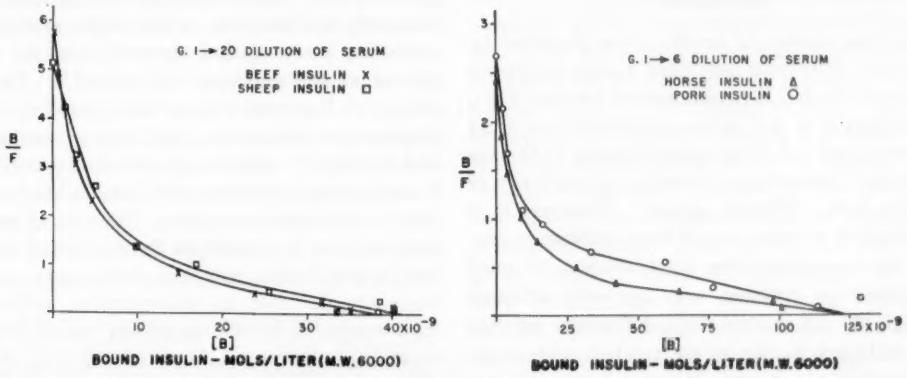

FIG. 5. SUBJECT G. RATIO OF BOUND TO FREE INSULIN (B/F) AS A FUNCTION OF THE CONCENTRATION OF BOUND INSULIN (B) FOR VARIOUS ANIMAL INSULINS

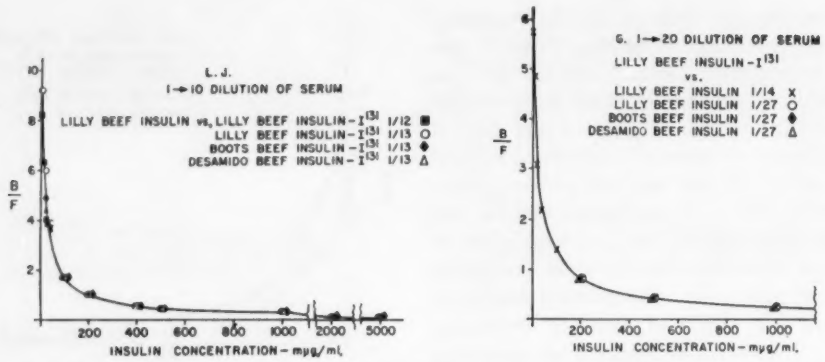

FIG. 6. B/F RATIOS FOR VARIOUS I^{131}-LABELED INSULINS AS A FUNCTION OF THE CONCENTRATION OF UNLABELED LILLY BEEF INSULIN IN L. J. (LEFT) AND B/F RATIOS FOR LILLY BEEF INSULIN-I^{131} AS A FUNCTION OF CONCENTRATION OF DIFFERENT PREPARATIONS OF UNLABELED BEEF INSULIN IN G. (RIGHT)

The three concentrations of Lilly (1/27), Boots and desamido (Boots) beef insulins tested in G. were in the range in which the most marked differences among the different animal species' insulins were observed (see Figure 2).

labeled Lilly beef insulin in the serum of L. J. (Figure 6).

Neither labeled A chain nor labeled B chain in trace concentrations was observed to bind to the *undiluted* serum of G., nor did these chains inhibit binding of beef insulin-I^{131} when present at concentrations of 250 μg. per ml. in antiserums with maximum insulin-binding capacities < 50 mμg. per ml.

Mixtures of the amino acids threonine, glycine and isoleucine (1 mg. per ml.) and of the dipeptide alanyl-serine (5 mg. per ml.) did not inhibit binding of beef insulin-I^{131} or pork insulin-I^{131}.

DISCUSSION

Previous studies of insulin cross reactions by techniques employing sensitized guinea pig uterus and anaphylaxis (10), complement fixation (11), neutralization in the mouse convulsion test (12) and sensitized red cell hemagglutination (13) have suggested immunological identity or similarity of insulins from different species. However, it is questionable whether any of these techniques permits precise quantitative determination of small differences in reaction with antibody although Lewis (10) did observe slight differences between beef and pork insulin in the reaction with sensitized guinea pig uterus. In some instances gross immunological differences among various insulins have been commented upon, *e.g.,* Moloney and Coval (12) found that certain preparations of guinea pig and sheep insulins were neutralized by homologous antiserums whereas the endogenous insulins were not neutralized. Also, Lowell (14) had observed that 30 units of human insulin was capable of inducing a decrease in blood sugar in human subjects resistant to a similar dose of crystalline beef-pork insulin and concluded that human and animal insulins were immunologically different. Lerman (15) correctly pointed out that the reaction of antiserum would be expected to be greater with insulins of other species than with insulin from the antiserum donor and emphasized that "any biological protection test is undoubtedly too crude to detect slight amounts of antibodies to an antigen derived from the same species as the antiserum is derived." The inability of Burrows, Peters and Lowell (16) to demonstrate competitive inhibition of binding of beef insulin-I^{131} by human insulin can be attributed to inadequate concentrations of human insulin used with insulin-resistant serums. The present studies have not only demonstrated the reaction of human insulin with human anti-beef, pork insulin serums but show also that, in concentrations sufficiently high compared to the maximum insulin-binding capacity of the serums, human insulin can occupy all the antibody combining-sites.

The similar total binding capacities for beef,

sheep, pork and horse insulins in individual serums and the ability of each species' insulin to inhibit completely, by competition, the binding of any other species' insulin indicate that the species-specific differences in binding and competitive inhibition are to be explained not on the basis of reactions with different antibody combining-sites but on the basis of energetically different reactions with the same antibody combining-sites. It is important to note here that the similarly reacting insulins show a closer chemical kinship with each other than with the dissimilarly reacting insulins. Thus beef insulin, with the sequence ala., ser., val. in the eighth, ninth and tenth positions, respectively, of the A chain, shares two amino acids in this site with sheep insulin (ala., gly., val.) whereas horse insulin (thr., gly., ileu.) shares two amino acids with pork insulin (thr., ser., ileu.).

The marked differences (in most antiserums) in direct and cross reaction of four different insulins which differ, at least insofar as amino acid sequence is concerned, only in the site comprising the eighth to tenth residues of the glycyl chain, indicate that this region is a site of antigenic determinacy. Since most commercial insulin preparations are approximately equal mixtures of beef and pork insulin it might be supposed that antibody specificity is directed toward the amino acids in the eighth to tenth positions of both proteins (independent of the possibility that other insulin sites may also be antigenic in man). It is not unreasonable to infer further that the degree of spatial complementarity [5] of the antibodies developed towards the ala., ser., val. sequence of beef insulin is greater than that of the antibodies directed against the thr., ser., ileu. sequence of pork insulin. However, both groups of antibodies are able to react with insulins of other species sharing *none* of the three particular residues of the original antigen (*viz.*, sheep insulin inhibits binding of pork insulin and horse insulin inhibits binding of beef insulin), although the energies of the reactions of the various insulins differ significantly. The greatest difference is seen between the reactions of horse insulin and of beef (or sheep) insulin with the "b" antibody sites. Here the equilibrium constants differ by a factor of 8 to 10 which indicates a difference of about 1.3 Kcal. in the standard free energy changes of the reactions. In general, the differences in reaction of the various insulins are greater with the "b" sites than with the "a" sites. This conclusion could be deduced also from the cross reaction experiments in which the differences were generally more marked at the higher insulin concentrations. Since the equilibrium constants (see Reference 4 for methods of evaluation) for horse insulin with "a" and "b" antibody sites are 3.1×10^8 L. per Mole ($\Delta F^\circ = -11.9$ Kcal.) and 3.4×10^6 L. per Mole ($\Delta F^\circ = -9.2$ Kcal.), respectively, the difference in ΔF° between the reactions for horse insulin (the weakest reacting animal insulin) and beef (or sheep) insulin still represents only a relatively small part of the total free energy change. However, a more extensive study might well reveal much greater differences in some antiserums.

Aside from the relatively high binding energy compared to that of other antigen-antibody reactions which has been commented on elsewhere (2), perhaps the most interesting aspect of the insulin-antibody system is the chemical nature of the reaction site on the antigen. In the case of sheep insulin, none of the amino acid R groups in the sequence 8 to 10 contains any permanently polarized group, *i.e.*, there are no charged (ionic) or hydrogen binding sites on the R groups although the N's of the peptide bond backbone undoubtedly have residual positive charges which would not be expected to differ among the various insulins. Furthermore, since sheep insulin reacts almost as well as beef insulin and more strongly than horse and pork insulins, little importance can be attributed to the hydroxyl groups of serine and threonine in the binding energy. It seems reasonable to conclude therefore that *at least* the *maximum differences* in energy of binding (and perhaps a significant fraction of the energy of binding) for the various insulins are attributable to van der Waals' forces alone, and that close spatial complementarity between antigen and antibody (rather than the attraction of oppositely charged polar groups) characterizes the site of this antigen-antibody reaction. Yet this conclusion cannot be made without reservation. If complementarity extends over an area consider-

[5] Neither this word nor "complementariness" is given in Webster's unabridged or the Century Dictionaries. The proper word appears to be complementarism. Nevertheless, "complementarity" is sufficiently entrenched in the scientific parlance as to justify its continued usage.

ably larger than the antigenic site itself, several polar groups of the insulin molecule might be involved in the reaction. The differences in reactivity among the different insulins might then simply be due to steric restrictions (the eighth and tenth residues in the A chains of horse and pork insulins have an extra methyl group over those of beef and sheep insulins) in a relatively small region of a larger reaction site that is bound to the antibody primarily by charged groups. According to this hypothesis, the antigenic determinacy which stimulates antibody formation may reside in a completely non-polar site but the antibody directed toward the antigenic site may be spatially and otherwise complementarily adapted to a larger surface of the antigen than the antigenic site itself through polar as well as non-polar linkages. Even if this were the case, however, the stronger binding of sheep than of horse insulin would have to be interpreted in terms of spatial complementarity. Another reservation would be concerned with the possibility that differences in the amino acid sequence 8 to 10 of the glycyl chain are accompanied by other differences in the helical configurations of the various insulins. However, in the absence of definite information on this point, further speculation is unwarranted.

The greater reaction of sheep insulin (which is not one of the original antigens) than of pork insulin (which is one of the original antigens) could obviously be explained by postulating that most (or all) of the antibody is directed toward beef insulin which bears a closer resemblance to sheep insulin than to pork insulin. However, there is no direct evidence bearing on this point and from preliminary studies in animals, in which single species' insulins are used as antigens, it appears as if this explanation may not be completely satisfactory. It should be emphasized strongly that these studies do not exclude a second antigenic site or a second site of reaction. Since the amino acid sequences are apparently elsewhere identical in all the different species' insulins employed here, their reactions at other sites would probably not be distinguishable in any event.

The absence of detectable reaction with labeled B chain was perhaps not unexpected. The lack of reaction of labeled A chain is to be explained on two bases. Firstly, this peptide is in a very much extended state as compared to its configuration within the intact insulin molecule since Craig (6) has found that on differential dialysis "it behaves like a molecule of changing molecular size mostly much larger than would be expected." Secondly, during the splitting of insulin the intra-A-chain disulfide bridge between 6 and 11 is broken with the formation of cysteic acid residues which might well provide insuperable obstacles to binding of the site in question. It is also of interest to note that I^{131}-labeled A chain alone of all insulin preparations (B chain included) fails to bind to paper strips but migrates faster than albumin on electrophoresis in veronal buffer (consistent with the acidic nature of the A chain).

SUMMARY AND CONCLUSIONS

1. Crystalline pork, sheep, horse, beef (two preparations) and beef desamido insulins and A and B chains of beef insulin were labeled with I^{131}.

2. The labeled insulin preparations were tested directly in reactions with insulin-binding antibodies in human anti-beef, pork insulin serums and were cross reacted in various combinations with unlabeled insulins of all species as well as with unlabeled human insulin.

3. No differences were observed in the extent to which the two preparations of beef insulin-I^{131} and desamido beef insulin-I^{131} reacted with insulin-binding antibodies or in the degree to which each of the unlabeled beef preparations competed against the binding of one of the I^{131}-labeled beef insulins.

4. In general, beef and sheep insulins were bound more strongly than pork or horse insulins in both direct and cross reaction studies. These similarities are paralleled by known chemical similarities among the different animal species' insulins. Human insulin cross reacted less strongly than did any of the animal insulins.

5. The observed differences and similarities in binding of the various animal insulins suggest that the site of differences in amino acid sequence (residues 8 to 10 of the A chain) constitutes at least part of the antigenic site as well as of the site of reaction with the antibody.

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NITROGEN CLEARANCE RATES OF RIGHT AND LEFT LUNGS IN DIFFERENT POSITIONS *†‡

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Uniform alveolar ventilation implies that during inspiration all alveoli simultaneously receive gas of the same chemical composition and of the same volume (relative to the previous preinspiratory volume) and that this mixes almost instantaneously with the preinspiratory alveolar gas. Studies with single or multiple inhalations of "reference" gases have shown that alveolar ventilation is nonuniform (uneven) in most normal men (2). The two principal theoretic explanations for this observation have been *a*) that inspired gases are stratified in the peripheral alveolar spaces or *b*) that different regions (central versus peripheral spaces of a lobe or segment; different lungs, lobes or segments) are ventilated at different rates. Although Roos, Dahlstrom and Murphy (3) again proposed the stratification theory, it was noted by Forster (4) that some of their experimental results could be equally consistent with a regional explanation.

The elimination rate (clearance) of pulmonary nitrogen (N_2) from the total lungs during inhalation of oxygen was found by Lundin (5) and by Svanberg (6) to be retarded in persons in the lateral decubitus position as compared with the rate in supine persons. The clearance curves of helium from the total lungs failed to demonstrate that alveolar ventilation was less uniform in the lateral decubitus than in the supine position (7). The present study was undertaken, in part, to determine whether analysis of N_2 clearance curves from the total lungs would clarify the nature of the retarded elimination of N_2 seen in the lateral decubitus position. Such studies on a few normal persons were sufficient to confirm the results of Lundin (5) and of Svanberg (6), and to indicate further that the retardation of complete elimination of N_2 was caused by both a reduction of alveolar dilution per breath and an increased degree of uneven ventilation.

In the lateral decubitus position, differences between the superior and the inferior lung in the clearance rates of alveolar gases were first shown directly in dogs (8) and later were shown (6) and calculated (9) to exist in man. In the present study, partial anatomic localization and further analysis of the uneven ventilation were obtained by measuring N_2 clearance curves and the respiratory dead space of individual lungs during bronchospirometry with the subjects in several positions. The results, which are in essential agreement with those reported by Svanberg (10), indicate that uneven ventilation exists within and between lungs, and that the latter difference is subject to postural variations.

Several theoretic but unrecognized alterations that have arisen in Lundin's modification (5, 10, 11) of the original method for analysis of pulmonary clearance curves reported by Fowler, Cornish and Key (12) also will be indicated.

PROCEDURES AND METHODS

Studies on total lungs. The subjects were five normal men 30 to 40 years of age, with an average height of 183 cm. (175 to 193 cm.) and weight of 82 Kg. (68 to 95 Kg.). After resting supine for 10 or more minutes, they breathed 99.5 per cent oxygen, using an open circuit, mouthpiece and nose clip, until the maximal N_2 concentration of end-tidal gas, as recorded continuously throughout each breath, was reduced to about 2 per cent. The expired volume was measured with a 120 L. spirometer. This was done with the subjects in the supine and the right and left lateral

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decubitus positions in varying orders. For three of the subjects, duplicate measurements in each position were made, and the concentration of expired N_2 and the expiratory flow also were recorded continuously, as noted below.

Studies on individual lungs. The subjects were five healthy male physicians 27 to 44 years of age and 179 cm. in average height. Premedication and introduction of a Carlens bronchospirometric catheter were done as outlined by Wright and Michelson (13), and the proper location of the catheter was confirmed by fluoroscopy. The subject first assumed the left lateral (three subjects) or the right lateral decubitus position, with one pillow supporting the head; subsequently, he assumed the supine and then the other lateral decubitus position. The leak-free placement of the Carlens catheter was tested before and usually after each change of position. The lungs were connected to the four-way valves of an oxygen-filled, double, closed circuit spirometer (Gaensler-Collins) via tubing and a two-channel rotary valve containing the sampling needle of a N_2 meter that was used to analyze gases passing to and from one lung. The closed circuits were modified with large bore, three-way stopcocks and valves so that, while room air was being breathed, each circuit could be converted to an oxygen-filled open circuit for later measurement of the pulmonary N_2 clearance rate. Gases expired from either lung on an open or closed circuit could be passed through a heated pneumotachograph similar to that of Lilly (14), a differential transformer-amplifier system being used as the transducer. In the initial lateral position and thereafter during changes of position, double closed circuit recordings were made to measure tidal volume and changes of the functional residual capacity. In the two lateral positions, N_2 clearance rates were measured for the dependent and the superior lung in turn. In the intervening supine position, the N_2 clearance rate was measured first on the previously dependent lung and then on the previously superior lung. Prior to any measurement of N_2 clearance, both lungs breathed room air until the end-tidal N_2 concentration was about 80 per cent; during each measurement of N_2 clearance, the opposite lung continued to breathe room air.

Continuous recordings of N_2 concentration and of expiratory flow were made with oscillographic galvanometers and a photokymograph at a paper speed of 25 mm. per second. The Lilly type of N_2 meter was calibrated for each subject with five known gas mixtures saturated at 37° C. It provided an accuracy of about ± 2 per cent N_2 at high concentrations, ± 1 per cent N_2 at concentrations from 10 to 30 per cent and ± 0.5 per cent N_2 at lower concentrations. In the studies on total lungs, the N_2 concentration of tidal gases was measured at the mouthpiece. With bronchospirometry, the sampling needle of the N_2 meter was located so that tubing dead spaces of 12 and 31 ml. existed between it and the Carlens catheter and the four-way valve, respectively. The mean N_2 concentration of gases expired during breathing of O_2 as "seen" by the N_2 meter was, therefore, greater than the true value, depending on the size of the tidal volume. The appropriate correction, averaging 14 per cent, was applied to values for lung volume calculated from mean expired concentrations and mean tidal volume. A measured correction of 0.1 second was applied to account for lag of the N_2 meter behind the simultaneous flowmeter recordings. Calibration of the flowmeter, from which the volumes pertaining to N_2 clearance curves during bronchospirometry were derived, was made for each subject from the area of flowmeter recordings of two or more expirations, obtained with simultaneous measurements of volume by the spirometer.

Pulmonary N_2 clearance rates were determined by the method of Fowler, Cornish and Kety (12). One modification was made in estimating the fractional concentration (F_b) of the N_2 in expired gas that was eliminated from the blood during bronchospirometry. This was necessary because N_2 was eliminated from the blood traversing the one lung that breathed O_2 but was added to the blood traversing the other lung that breathed air. If it is assumed that half the cardiac output ($\dot{Q}$) traverses each lung and that the alveolar pN_2 of the "O_2 lung" is initially reduced abruptly to near zero, this lung initially will eliminate N_2 from the blood at a maximal rate equal to 0.5 ($\dot{Q} \times C_{\bar{v}}$), where $C_{\bar{v}}$ equals the N_2 concentration in mixed venous blood when the body is in N_2 equilibrium with atmospheric air. Thereafter, the rate of elimination of N_2 by this lung will decrease to a minimal constant value of 0.25 ($\dot{Q} \times C_{\bar{v}}$), which occurs when the mixed venous concentration equals 0.5 $C_{\bar{v}}$. This equilibrium is reached when the rate of uptake of N_2 by the "air lung" equals the rate of elimination by the "O_2 lung." The amount (milliliters) of N_2 eliminated when both lungs breathe O_2 for seven minutes, called here the "Cournand factor" (15), approximately equals 0.5 ($\dot{Q} \times C_{\bar{v}}$) $\times$ 7 minutes. Therefore, the correction factor used here, assuming the minimal constant elimination rate for the "O_2 lung" was F_b, equals 0.5 Cournand factor divided by the number of breaths in seven minutes $\times V_T$, where V_T is tidal volume. This correction is appropriate for the later parts of the pulmonary N_2 clearance, when alveolar N_2 concentrations are small; it will cause only negligible error in the early parts, when alveolar N_2 concentrations are large.

In several instances, progressive changes in tidal volume occurring during N_2 clearance precluded an accurate analysis of the total clearance curve. In these instances, the functional residual capacity was measured by summating the amounts (milliliters) of N_2 eliminated on successive individual expirations (corrected for the N_2 of blood and inspired gas) until the expired alveolar concentration was 5 per cent or less and dividing by the change of alveolar concentration, as by the method of Darling, Cournand and Richards (16).

The respiratory dead space for each lung was recorded as the average of measurements on two or more expirations, obtained from simultaneous records of N_2 concentration and flow, using the "estimation" method of Fowler (17). The subcarinal dead space was obtained by substracting 22 and 29 ml. from the measured values for

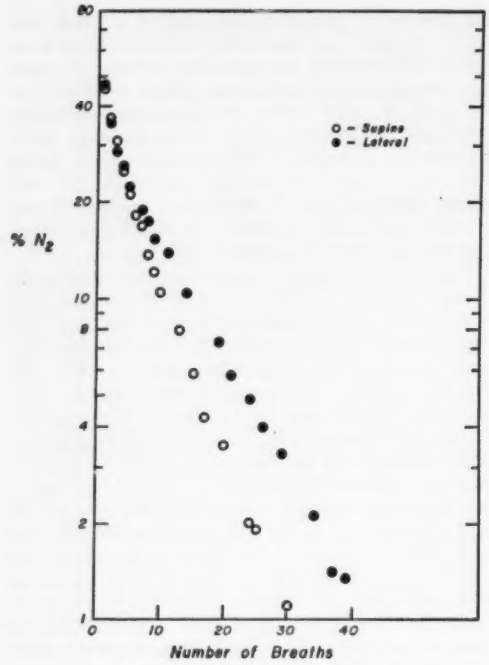

FIG. 1. MEAN CONCENTRATION OF EXPIRED N_2 DURING CLEARANCE FROM BOTH LUNGS

In the supine and right lateral decubitus positions, respectively, tidal volume was 550 and 537 ml., and functional residual capacity was 2,170 and 2,700 ml.

right and left lungs, respectively. These corrections represented the actual volume of channels of tubing and catheter (27 ml. on the left and 24 ml. on the right), with a 5 ml. adjustment estimated to account for the asymmetric location of the catheter in the carinal region. The total dead space, as used in certain calculations, was the measured value plus 31 ml. of distal tubing.

RESULTS

Total lungs

Typical clearance curves are illustrated in Figure 1 and the results of their analysis are given in Table I. The tidal volumes in Table I were measured spirometrically, but the values for functional residual capacity and dead space were obtained by analysis of clearance curves. All clearance curves, plotted semilogarithmically, were nonlinear, but they could be resolved into two or three components, representing fractions of the functional residual capacity having different clearance or replacement rates.[1] When the subject was supine, the more slowly ventilated component comprised about 80 to 90 per cent of the functional residual capacity; its fractional replacement per breath was about 10 per cent. For the total lungs, molecules of N_2 remained for an average of about eight breaths, which was 24 per cent longer (delay in pulmonary clearance) than if alveolar ventilation had been uniform. The concentration of N_2 in end-tidal gas was reduced to 5 per cent and 2 per cent in 1.8 and 2.8 minutes, respectively.

Results for the two lateral positions were combined, since consistent differences were not apparent. Here, the complete clearance was less rapid than it was in the supine position, as shown by significantly different mean values for both the time and the volumes required to reduce the end-tidal concentration of N_2 to 5 and 2 per cent, and by values derived from analysis of the clearance curves (Table I). These values indicated that the slower clearance was the result of *a*) reduction of

[1] The method used for analysis of N_2 clearance curves (12) is based on equations for a cyclically ventilated system of one or more bellows, within each of which ventilation is uniform, and from which the separate expired tidal volumes are combined. The N_2 concentration within any one bellows will be reduced on each successive breath of O_2 to a fraction of its preceding value, designated as w, the alveolar dilution factor, when

$$w = V_L \div V_L + (V_T - V_D) \text{ and}$$

where V_L is the volume in which N_2 is contained at the end of expiration, V_T is the tidal volume and V_D is the volume of the dead space. The fractional replacement per breath equals 1-w. If there are two or more bellows or regions having different clearance rates, their alveolar dilution factors are w_1, w_2 and so forth, and f_1 and f_2 denote their respective decimal fractions of the total end-expiratory volume. Experimentally, the mean expired N_2 concentration of successive breaths is measured and the semilogarithmic plot (Figure 1) is resolved graphically into one or more components. From the slopes and intercepts of the components, values of w and f are obtained; from these values, one can calculate the average interval during which molecules of N_2 remained in the total system (actual average number of breaths), thus characterizing the clearance rate of the total system, and also the average interval during which molecules of N_2 would remain in a uniformly ventilated system having the same total tidal volume, dead space and functional residual capacity as the components (ideal average number of breaths). A delay in pulmonary clearance indicates the extent to which complete clearance of N_2 is retarded by the presence of uneven ventilation, that is, different replacement rates of various regions.

TABLE I

*Pulmonary N_2 clearance of total lungs in supine and lateral decubitus positions**

Row†	Factor	Supine Value	Supine No.	Lateral decubitus Value	Lateral decubitus No.
1	End-tidal F_{N_2} to 0.05‡ *min.*	1.83 ± 0.16	8	2.86 ± 0.16	15
	L.	13.1 ± 1.5	8	18.9 ± 1.1	15
2	End-tidal F_{N_2} to 0.02‡ *min.*	2.79 ± 0.23	8	4.12 ± 0.22	15
	L.	19.7 ± 1.6	8	27.3 ± 1.6	15
3	Tidal volume, *ml.*	625 ± 65	8	602 ± 46	15
4	Functional residual capacity (F.R.C.), *ml.*	2,160 ± 246	6	2,950 ± 274	11
5	Dead space, *ml.*	170 ± 18	6	155 ± 11	11
6	Respirations per min.	12.2 ± 1.1	8	11.8 ± 0.7	15
7	Fractional replacement per breath $(1 - w_2)$ of rapidly ventilated component§	0.40 ± 0.034	6	0.34 ± 0.028	9
8	Fractional replacement per breath $(1 - w_1)$ of slowly ventilated component‡	0.114 ± 0.005	6	0.069 ± 0.03	11
9	Slowly ventilated component fraction (f_1) of F.R.C.	86.3 ± 2.8	6	86.1 ± 1.9	11
10	Actual average number of breaths‡	8.13 ± 0.29	6	13.4 ± 0.62	11
11	Ideal average number of breaths‡	6.62 ± 0.32	6	9.15 ± 0.41	11
12	Pulmonary clearance delay, %‡	24 ± 4.1	6	47 ± 4.1	11

* Volumes are BTPS (body temperature, ambient pressure, saturated). Values are mean ± standard error of mean. No. is number of observations.

† Definition of Rows 7 through 12 are given in text Footnote 1.

‡ Mean values differ significantly. Interindividual variation in functional residual capacity produces nonsignificant difference in the group mean, but all lateral values exceed supine values for the same subject.

§ Two of the lateral decubitus curves contained a small third component (w_3).

fractional replacement per breath for both the more slowly ventilated component (smaller value of $1\text{-}w_1$) and for the total lungs (larger ideal average number of breaths), the latter being caused by an increase of functional residual capacity without a corresponding increase of effective tidal volume, and *b*) an increase in the nonuniformity of ventilation (increased delay in pulmonary clearance).

Despite the considerable increase of end-inspiratory lung volume in the lateral positions, a significant change in the dead space was not ob-

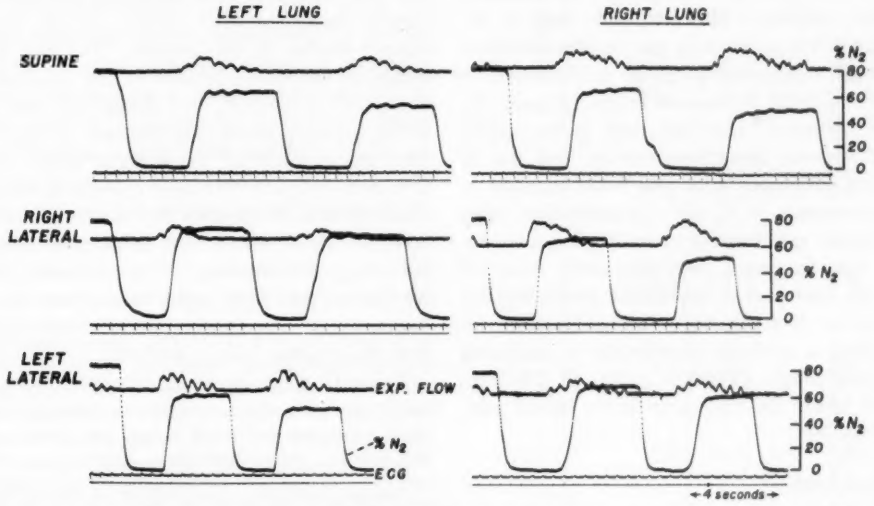

FIG. 2. CONCENTRATION OF N_2 AND EXPIRATORY FLOW OF INDIVIDUAL LUNGS DURING FIRST TWO BREATHS OF OXYGEN BY ONE SUBJECT

Records read from left to right. See text.

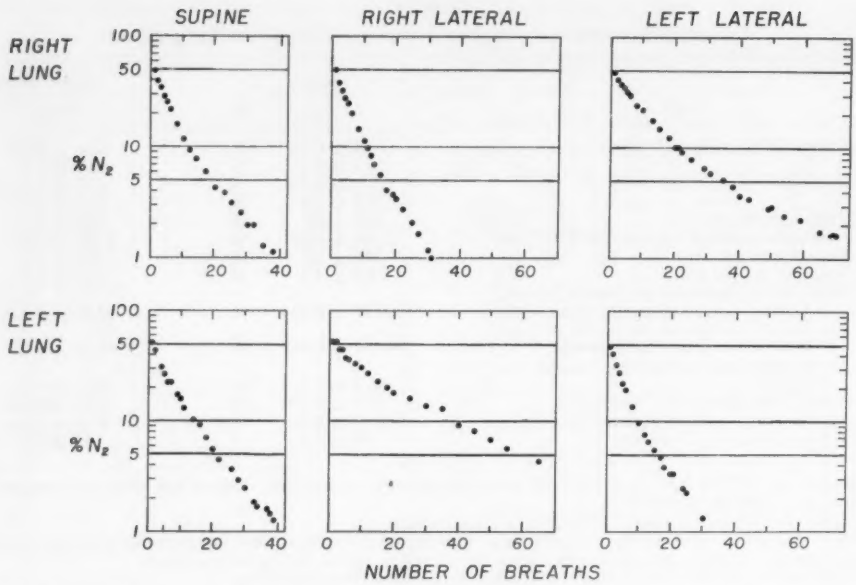

FIG. 3. MEAN CONCENTRATION OF EXPIRED N_2 DURING CLEARANCE FROM INDIVIDUAL LUNGS OF ONE SUBJECT IN DIFFERENT POSITIONS

served. However, calculation of the dead space from clearance curves is of limited accuracy (12). Simultaneous records of the concentration of expired N_2 and flow, needed for measurement of dead space on individual breaths (17), were available for three subjects. However, this method requires that expired alveolar gas be identifiable as a "plateau" of concentration, as is illustrated in the records from individual lungs (Figure 2). Suitable "plateaus" were observed in the supine position without bronchospirometry, but not in the lateral positions. After the initial increase in the concentration of N_2 early in expiration, large and irregular variations of concentration, both increases and decreases, were frequently observed during the later part of expiration, precluding the identification of a plateau. This presumably resulted from a variable asynchrony of emptying of the two lungs. Further study of this phenomenon and of the dead space in the lateral positions is needed.

Individual lungs

Figure 2 shows typical recordings of the first two expirations while the subject breathed oxygen in various positions. In each expiration, the "alveolar plateau" showed a progressive variation in N_2 concentration, qualitatively indicating uneven alveolar distribution of inspired O_2. Also noted were variations in alveolar N_2 concentration and expiratory flow occurring periodically at cardiac frequency (18); these will not be considered further in this report. The rate of decrease on successive breaths of the concentration of expired alveolar N_2 was similar for both lungs in the supine position, but the rate in the lateral positions was less for the superior lung. Figure 3 presents typical corrected curves of the mean concentration of expired N_2 vs. the number of breaths. These curves were in all cases nonlinear, indicating the presence of nonuniform alveolar ventilation, and they could be resolved into two components. The slower rate of clearance of N_2 from the superior lung is evident.

Table II presents mean values derived from analysis of the clearance curves and also values of the respiratory dead space measured on individual breaths by the "estimation method" (17).

Supine position. The volume of the right lung was larger than that of the left, averaging 57 per cent of the total functional residual capacity. The right lung contained about 62 per cent of the total

subcarinal dead space. However, the greater tidal volume of the right lung, about 55 per cent of the total, and its distribution were such that the average clearance rates of the two lungs were similar, as were the clearance rates of the rapidly and the slowly ventilated components. The latter comprised about 80 per cent of the functional residual capacity of each lung. The average interval during which molecules of N_2 remained in the lungs was about one minute, corresponding to about 10 breaths, or a 10 per cent replacement of alveolar gas per breath. This was about 30 per cent less rapid than it would have been if alveolar ventilation had been uniform.

Lateral positions. When the subjects changed from the supine position to either of the lateral decubitus positions, the functional residual capacity of the dependent lung changed relatively little, whereas that of the superior lung increased by more than 0.5 L. over its supine value. The average increase recorded by closed circuit spirometry was 680 ml. Consistent changes in the dead space of either lung were not observed.

When the subject was in a lateral decubitus position, the dependent lung uniformly received a greater percentage of both total and effective tidal volume than did the same lung when the subject was supine.[2] The clearance rate of the right lung was slightly more rapid when dependent than when supine, as indicated by smaller values of the actual average number of breaths in four subjects and by a more rapid reduction of the end-tidal N_2 concentration in a fifth subject, whose clearance curves were not all suitable for graphic analysis. More rapid clearance was achieved by small increases in the fractional replacement per breath and in the respiratory frequency (about one breath per minute).

The clearance rate of the left lung was not con-

[2] The measurements in Table II were not obtained simultaneously on the two lungs. Simultaneous measurement by spirometry showed the percentage of total tidal volume received by the right lung to average 54, 43 and 64 per cent for the supine, left lateral and right lateral positions, respectively, which corresponds with the consecutively measured tidal volumes in Table II. The percentage of effective tidal volume of the dependent lung increased when it was calculated from the total dead space of each lung and tidal volumes recorded either simultaneously or as in Table II.

TABLE II

*Pulmonary N_2 clearance of individual lungs**

Position	Lung	Tidal volume	Sub-carinal dead space†	Resp. per min.	Funct. residual capacity (F.R.C.)	Time for end-tidal N_2 to decrease to 5%	Ventilatory components: Fractional replacement per breath $1-w_1$	Ventilatory components: Fractional replacement per breath $1-w_2$	Ventilatory components: Slower ventilated fraction of total F.R.C. (f_1)	Average interval N_2 molecules remained: No. breaths Actual	Average interval N_2 molecules remained: No. breaths Ideal	Average interval N_2 molecules remained: No. min. Actual	Average interval N_2 molecules remained: No. min. Ideal	Pul. N_2 clearance delay
		ml.	*ml.*		*ml.*	*min.*								%
Supine	L	285	35	11.1	1,170	2.7	0.097	0.338	0.84	9.2	7.0	1.03	0.79	32
	R	378	54	11.1	1,485	2.6	0.090	0.305	0.80	10.0	7.6	1.00	0.75	32
Left Lateral	L	344	36	12.7	1,090	2.1	0.105	0.304	0.79	9.6	7.6	0.90	0.71	26
	R	313	56	12.1	2,205	5.2	0.054	0.196	0.84	17.2	13.0	1.74	1.33	29
Right Lateral	L	178	40	12.3	2,060	8.4	0.031	0.142	0.91	28.4	22.8	2.67	2.10	25
	R	370	46	12.2	1,458	2.2	0.098	0.272	0.82	9.6	7.9	0.83	0.68	22

* Volumes are BTPS (body temperature, ambient pressure, saturated).
† Values of dead space represent measurements on individual breaths.

sistently different when it was supine and when it was dependent.

The clearance rate in superior lungs was uniformly slower than it was in the same lungs when supine. In six instances, the average interval during which molecules of N_2 remained was one minute in the supine position, whereas this was increased in the superior position to mean values of 1.7 and 2.7 minutes for the right and left lungs, respectively. In four instances in which clearance curves could not be analyzed, reduction of the end-tidal concentration of N_2 to 5 per cent required two to three minutes in the supine position and five to 11 minutes in the superior position. In four of five subjects, the clearance rate of the superior lung was slower for the left lung than for the right. These retarded clearance rates were largely the result of reduction of effective tidal volume of the superior lung, which was particularly pronounced for the left lung. Fractional replacement per breath was reduced for both the rapid and slow components.

Consistent variations with changes in position were not observed in the fractional volume of the slowly ventilated component or in the magnitude of nonuniform ventilation within an individual lung, as shown by values for delay in pulmonary clearance.

DISCUSSION

The finding that complete N_2 clearance from the total lungs is slower in the lateral decubitus than in the supine position and is produced largely by a reduced clearance rate of the more slowly ventilated fraction agrees with the results of Lundin (5) and Svanberg (6, 10). However, Blair and Hickam (7), using helium clearance curves, did not observe such a difference in the most slowly ventilated space and did not find that ventilation was less uniform in the lateral position.

The different methods used are probably responsible for these varying results. The limited analytic range for helium permitted measurement of only the terminal 6 per cent of the total clearance curve, probably making it difficult to detect differences that become apparent when the total clearance curves can be described, as in Figure 1.

The clearance curves recorded from individual lungs by bronchospirometry were largely similar to those of Svanberg (10), except that in the present bronchospirometric studies the clearance times were longer than those of Svanberg's subjects and of our subjects studied without bronchospirometry. This retardation was apparently the result of reduced minute volumes attributable to medication. Our subjects undergoing bronchospirometry received not only morphine, as did Svanberg's, but also 1.5 grains of secobarbital (Seconal®) and at times were nearly asleep.

Svanberg observed, as we did in this study, that functional residual capacities were larger with bronchospirometry than without, and he suggested that the resistance to flow imposed by the catheters was responsible (10). However, a pause between the end of expiratory flow and the start of inspiration, as estimated from the N_2 recordings (Figure 2), was commonly seen. This would indicate that elastic rather than resistive forces were determining the end-expiratory volume. The larger functional residual capacities observed during bronchospirometry may be the result of the multiple systemic and tracheobronchial medicaments used. Our data, and those of Svanberg (10), show that the average ratio of total tidal volume to total functional residual capacity was several per cent smaller with bronchospirometry than it was without. Thus, the fractional alveolar replacement per breath normally may be slightly greater than that occurring during bronchospirometry. The absolute values of the dead space of individual lungs also must be taken with some reservation, in view of possible alterations by the drugs employed.

The bronchospirometric results indicate that at least four different alveolar clearance rates may exist throughout the lungs, particularly in the lateral positions. This was thought previously to be probable (12, 19). Our inability to resolve more than two or three components in N_2 clearance curves of total expired gases is caused largely by limitations in defining the terminal parts of the curve, where the slowest components must be separated but where analytic accuracy for N_2 has been limited and where errors in the estimated correction for blood N_2 may have a relatively large effect. The terminal curve probably can be defined better with helium analyses, and results of such studies (7) suggest that fractional replacement rates even smaller than 3 to 4 per cent per

breath, noted in the superior lungs in this study, may exist normally.

Other current problems in the analysis of N_2 clearance curves may be noted. When the method of Fowler and associates (12) was modified by Lundin, Svanberg, Bouhuys and Hagstam (5, 10, 11), several apparently unrecognized consequences were introduced. The modification was that of measuring end-tidal concentration rather than the mean expired concentration as used originally (12). The use of the latter, which is a more difficult measurement, was proposed because the expired alveolar concentration may vary within the course of a single expiration, particularly with pulmonary disease, whereas only one value exists for mean expired concentration. The modification was justified by Lundin and co-workers for use in normal subjects by the experimental finding of similar values of dilution factors and fractional volumes obtained from either end-tidal values or the equivalent of mean expired concentrations. We also have found that this is generally true for normal subjects. However, by using the definitions and assumptions of the original theoretic treatment (12), one can derive several differences. In a uniformly ventilated system, either the mean expired concentration or the alveolar concentration may be used with identical results. However, in an unevenly ventilated system, the original method yields the end-expiratory fractional volumes having particular dilution factors, whereas the use of alveolar concentrations and the modified algebraic treatment (5) yields the end-inspiratory fractions, provided that the alveolar concentration selected equals the mean expired alveolar concentration. If it does not, the derived end-inspiratory fraction will be incorrect. By definition, the end-expiratory fractional volume of a relatively underventilated component is greater than its end-inspiratory fraction. Further values derived from fractional volumes and dilution factors also differ. The use of end-inspiratory fractions in the original equations (12) yields smaller values for *a*) the average number of breaths during which molecules of N_2 remain in the system and *b*) the number of breaths during which molecules of N_2 would have remained if ventilation had been uniform. The calculated value for delay in pulmonary clearance is also altered.

Although uneven alveolar ventilation has been known to exist for a long time, its nature (alveolar stratification vs. regional variations) and anatomic location are becoming apparent only currently. The difference in clearance rates between lungs in the lateral positions represents a regional type of uneven ventilation, and it results at least in part from differing percentage volume changes of the two lungs. Uneven ventilation within single lungs also is present. It has been found that N_2 clearance is more rapid in the right lower lobe than in the right upper lobe of healthy persons in the erect position (20), and evidence of uneven intralobar ventilation has been found in man (20) and dogs (8). The variations between lungs with change in position show that relatively retarded alveolar ventilation is not restricted to a particular anatomic location. The responsible thoracic mechanics requires further study, although the ventilatory implications of the anatomy of the lungs and bronchi are becoming more evident (21, 22).

SUMMARY

Healthy subjects breathing oxygen had a slower rate of clearance of pulmonary nitrogen in the lateral decubitus position than when they were supine. Analysis of clearance curves showed this retardation to be the result of two factors. First, the total functional residual capacity was increased without an accompanying increase of total effective minute volume. Secondly, the proportion of the total effective minute volume that was distributed to the underventilated fraction (about 85 per cent) of the functional residual capacity was further reduced in the lateral positions.

A partial anatomic localization of the variously ventilated regions was attempted by measuring nitrogen clearance rates and the dead space of individual lungs of five normal men in the supine and both lateral decubitus positions by use of bronchospirometric catheters. Clearance curves of multiple exponential form were found, as Svanberg has noted, indicating the presence of uneven ventilation within individual lungs in all positions.

In the supine position, the tidal volume, dead space and functional residual capacity were greater for the right lung than for the left, but the clearance rates and relative magnitude of slowly and

rapidly ventilated regions of both lungs were similar.

In lateral decubitus positions, the ventilatory pattern of the dependent lung was similar to that of the same lung in the supine position. The superior lung had an increased functional residual capacity and a decreased tidal volume. The clearance rates of both rapidly and slowly ventilated components of a lung were only half as great when it was in the superior position as they were in the same lung when the subject was supine.

The slow clearance rates of the superior lung appear to be largely responsible for the retardation of nitrogen clearance from the total lungs observed in lateral decubitus positions. This represents a regional type of uneven alveolar ventilation. The variations between lungs with change in position show that relatively retarded alveolar ventilation is not always restricted to a particular anatomic location.

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AN EXPERIMENTAL EVALUATION OF THE INDICATOR DILUTION TECHNIQUE FOR THE MEASUREMENT OF MITRAL REGURGITATION *

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In the selection of patients for mitral commissurotomy, accurate assessment of the amount of mitral regurgitation on a clinical basis may be extremely difficult (1). Hence, the indicator dilution technique, which offers some possibility for detection and a quantification of the regurgitant flow, has been widely applied to this problem in patients (2–5). However, as much of the work has been done in patients in whom precise quantification of the amount of insufficiency was not possible, experimental proof of the value of the technique in detecting known amounts of regurgitation is lacking. For this reason it was felt to be important to evaluate the technique in an experimental preparation in which the regurgitant flow could be controlled and measured. To do this, regurgitation was produced in the open-chested dog by an external polyvinyl shunt between the left ventricle and the left auricle. With this preparation, multiple comparisons between curves obtained with the shunt open and closed could be made, the amount of regurgitation could be measured, and yet blood flow and volume were not artificially fixed as in a model.

MATERIAL AND METHODS

Twenty-five adult mongrel dogs weighing between 9 and 24 Kg. were studied. The data from 15 of the dogs were considered satisfactory and used in the study. Chloralose 50 mg. per Kg. and urethane 500 mg. per Kg. were used for anesthesia in all cases, and respiration was controlled using intermittent positive pressure with 100 per cent oxygen through a No. 32 endotracheal tube. The chest was opened through the left fifth interspace and the pericardium opened widely. A No. 7 Cournand catheter was introduced into the main pulmonary artery and polyvinyl catheters inserted into the left auricle and left ventricle. Both catheters were 22 cm. in length; the left auricular catheter was 1.2 mm. internal diameter and 1.8 mm. outer diameter, the ventricular catheter was 0.6 mm. internal diameter and 1.0 mm. outer diameter. Pressures were recorded using a Hathaway blood pressure control unit, Type MBC2 with variable inductance gauges and a Hathaway oscillograph, Type S-14-C. Pressures were recorded before the shunt was put in and before and after all cardiac output indicator dilution determinations, both with the shunt open and closed. Systolic, diastolic and mean pressures were measured for the left ventricle and the pulmonary artery, and mean pressure for the left auricle. Mean pressures were determined planimetrically in all cases.

The shunt consisted of a polyvinyl tube, 4 mm. internal diameter and 31 cm. long with a "Y" tube in the middle, so that a side tube could be led off and attached to a reservoir. The shunt was introduced into the left auricle after the tip of the left auricular appendage had been removed; the other end was introduced into the left ventricle through a stab wound in the apex and both were fastened in by purse-string sutures. (Figure 1 shows the shunt in place.) To measure shunt flow, both limbs of the shunt, that is, to the left auricle and to the reservoir were unclamped, the reservoir was set at left auricular level and the limb leading into the auricle was clamped. Flow was then measured into the reservoir for 10 seconds or 50 ml. This amount of blood loss did not alter blood pressure and blood was immediately returned to the dog. Shunt flow was measured before and after all cardiac output determinations done with the shunt open. With this size shunt, the regurgitant flow varied between 20 and 38 per cent of the forward flow, forward flow being measured by the dye dilution technique.

Cardiac outputs were done using the dye dilution method of Stewart and Hamilton (6) with 5 μc. of radioactive iodinated human serum albumin in 0.5 ml. of saline as the indicator. The amount injected was determined by weighing the syringe before and after injection. For the left auricular (LA) injections, a saline-filled, 2 ml. capacity polyethylene tube was attached to the auricular catheter. This was filled with the indicator through one limb of a three-way stopcock, and then the contents rinsed in as rapidly as possible with a 10 ml. saline rinse through the other limb of the stopcock. Sample collection was begun with the start of the rinse which was considered zero time. The duration of the rinse was one to two seconds. Interrupted samples timed by a metronome were taken from the femoral artery at one second intervals using an 18 cm. length of polyethylene tubing (3 mm. internal diameter

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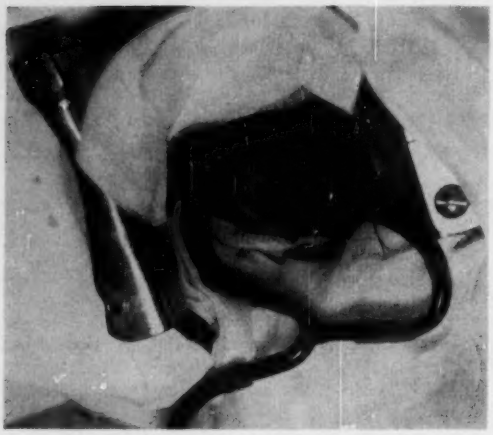

FIG. 1. SHUNT IN PLACE IN THE DOG

and total capacity 1.05 ml.) attached to an indwelling No. 18 Cournand needle. The duration of the sampling was 30 seconds and the volume of each sample varied between 0.5 ml. and 1 ml. Blood loss was replaced after each output, using blood from a donor dog obtained in advance. Five-tenths ml. samples were pipetted into distilled water, mixed and counted in a well counter for one minute. Counts were then plotted on semi-log paper, the downslope extrapolated to 5 per cent of the peak concentration and the curve then replotted on linear graph paper so that the area of the curve could be calculated. Cardiac output was calculated by the usual Stewart-Hamilton formula (6); $COP = \frac{I\ 60}{\bar{c}\ t}$, where I = amount injected in counts per minute, $\bar{c}$ = mean concentration in counts per minute per liter, and t = time of curve. Slope was calculated from the formula $\frac{\text{Log e C1} - \text{Log e C2}}{T2 - T1}$, where C1 and C2 are concentrations in counts per minute per liter and T1 and T2 are the comparable times on the downslope of the curve. The concentrations, peak (PC), least (LC) and recirculation (RC), and the times, appearance time (AT), disappearance time (DT) and build-up time (BT) are those described by Wood and Swan (7). Their derivation is shown in Figure 2. Disappearance time was taken as the time from the peak concentration to 5 per cent of the peak concentration. Mean circulation time was calculated using the formula: $M.C.T. = \frac{\Sigma\ (CT)}{\bar{c}\ t}$, where C = counts per minute at time T, T = time interval in seconds from the time of injection until the time at which C is measured, $\bar{c}$ = mean concentration and t = time of curve.

Central blood volume was calculated as $C.B.V. = \frac{M.C.T. \times COP}{60}$. Slope volume was calculated by the method of Newman and co-workers (8):

$$S.V. = \frac{\text{cardiac output (ml. per second)}}{\text{slope}}.$$

The sequence of the output measurements was varied, so that in some dogs an output was done first with the shunt open and repeated after the shunt had been clamped for 10 minutes, and in others the procedure was reversed. In six dogs duplicate measurements were made. In seven dogs the reproducibility of cardiac output measurements with the shunt open and using LA injection was studied using identical injections 10 minutes apart. In nine dogs, dye output determinations were also obtained by injection into pulmonary artery both with the shunt open and closed, to compare outputs from two different injection sites.

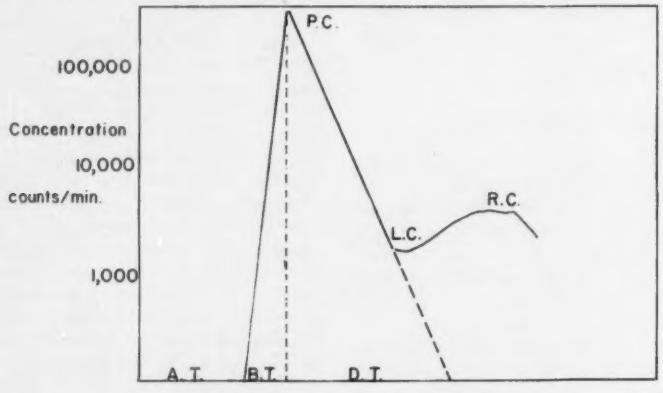

FIG. 2. TIME AND CONCENTRATION COMPONENTS OF THE DYE DILUTION CURVE

TABLE I

Data from dye dilution curves in 15 dogs

Dog No.	Left atrial pressure		Cardiac output		Mean circulation time		Central blood volume		Slope		Slope volume	
	mm. Hg		*L./min.*		*sec.*		*ml.*				*ml.*	
	O*	C*	O	C	O	C	O	C	O	C	O	C
5	6	7	1.08	1.13	5.7	4.9	103	92	0.72	0.85	25	22
6	8	6	0.94	1.03	10.1	10.0	158	180	0.49	0.49	32	26
7	4	3	0.56	0.68	6.3	5.7	59	65	0.30	0.98	31	12
8	7	6	1.20	1.38	7.5	7.7	151	178	0.56	0.79	36	29
9			1.13	1.15	10.2	6.2	192	119	0.32	0.62	60	31
10	9	6	0.90	0.73	6.4	7.1	96	87	0.35	0.54	42	22
11	7	6	0.85	0.84	6.7	5.9	95	83	0.55	0.98	26	14
16	4	3	1.56	1.33	9.1	9.9	236	230	0.59	0.81	44	27
17	5	5	0.67	0.71	10.1	9.2	112	109	0.31	0.36	31	33
19	5	6	1.06	1.03	12.8	11.0	226	188	0.33	0.49	54	35
20	8	7	1.16	1.16	11.7	12.7	227	245	0.42	0.44	52	44
21	2	1	1.50	1.40	7.2	7.6	180	177	0.47	0.64	53	36
22	3		2.46	2.87	7.7	6.7	316	322	0.54	0.73	78	65
23	7	4	0.78	0.82	10.0	7.6	129	104	0.34	0.48	38	29
24	4	3	2.5	2.3	8.3	7.9	345	286	0.49	0.59	78	68
Mean	5.6	4.8	1.22	1.23	9.0	8.0	175	168	0.45	0.65	45	33
S. D. ±					±2.3	2.1	82	80	0.12	0.19	21	16

* O = shunt open; C = shunt closed.

RESULTS

The basic data from the 15 dogs are shown in Tables I and II. In these tables, only the first set of outputs with the shunt open and closed was used. However, in six of the dogs multiple determinations were done and used in some of the statistical analyses. The simultaneous changes in pressure in the auricle and ventricle when the shunt was clamped are shown in Figure 3. In all cases there was an abrupt fall in left atrial pressure. Mean atrial pressure with the shunt open was 5.6 mm. Hg and closed, 4.8 mm. Hg. The left ventricular pressure demonstrated an immediate rise with closing the shunt and shortly therefater a fall to the preclosure level. Cardiac

TABLE II

Data from dye dilution curves in 15 dogs

Dog No.	Appearance time		Build-up time		Disappearance time		Peak conc.		Least conc.		Recirculation conc.		Shunt flow % of forward flow
	sec.		*sec.*		*sec.*		*cpm/L.*		*cpm/L.*		*cpm/L.*		
	O*	C*	O	C	O	C	O	C	O	C			
5			2	3	6	5	28.5	26	0.7	0.5			
6			4	3	12	10	47	56	0.8	0.8			
7	2	3	3	3	10	4	52	80	2.2	0.9			
8	4	4	3	3	10	8	17	16	0.3				
9	6	3	2	2	11	8	56	82	2.2	1.3			
10	3	3	2	4	11	12	50	56	1.1	0.5			
11	3	3	3	2	8	8	51	57	1.3	0.7			38
16	5	6	3	3	7	7	31.8	40.9	1.2	1.1			23
17	5	5	4	3	12	11	32.9	50.4	1.6	1.7			35
19	7	6	4	4	16	16	22.5	23.3	0.65	0.39	O	C	
20	6	7	4	5	14	12	35	47.4	1.07	0.93	4	4	36
21	4	4	3	3	9	8	34	48	1.4	0.75	5.4	7	20
22	5	4	3	2	7	7	27.5	32	1.2	0.91	3.8	4.6	25
23	4	4	3	3	10	9	54.1	69.8	3.4	2.9	9	10	21
24	3	6	3	2	9	7	30	33	0.9	0.8	5.2	6	28
Mean	4.4	4.6	3.0	3.0	10	9	37.9	47.8	1.3	0.94	5.4	6.3	
S. D. ±							12.4	19.8	0.77	0.65			

* O = shunt open; C = shunt closed.

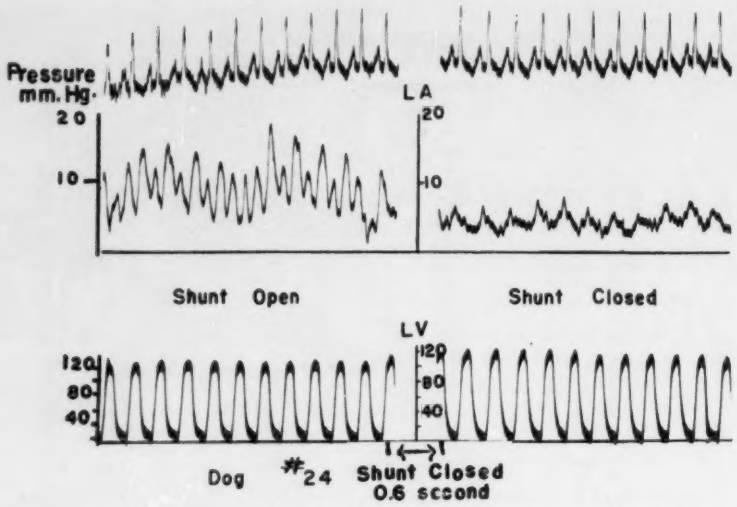

FIG. 3. ACUTE CHANGES IN LEFT AURICULAR AND VENTRICULAR PRESSURE WITH CLOSING THE SHUNT

output with the shunt open and closed as measured by the dye dilution method was the same, the ratio of open to closed being 0.99 (S.D. ± 0.12). In nine dogs, with the shunt open, the output obtained by injection into the pulmonary artery was compared to that from the left auricle. The mean ratio of pulmonary artery to left auricle was 0.89 (S.D. ± 0.13). The reproducibility of the left auricular injection outputs was determined by two separate output measurements 10 minutes apart with the shunt open. The ratio of the first to the second output was 1.00 (S.D. ± 0.18).

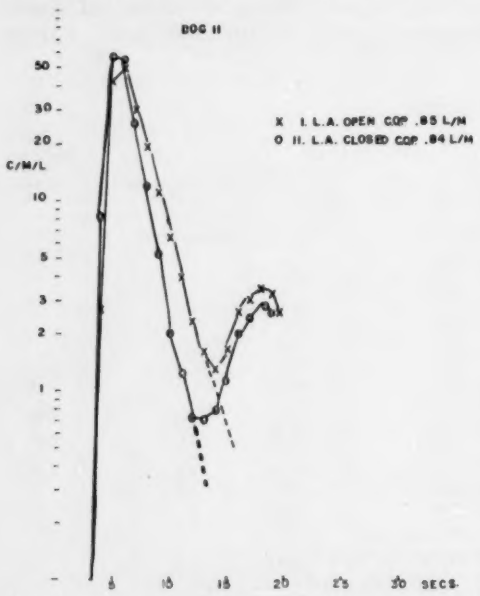

FIG. 4. TYPICAL DYE DILUTION CURVES OBTAINED WITH THE SHUNT OPEN AND CLOSED IN DOG NO. 11

For all the dogs studied, the curve obtained with the shunt open could be easily separated from that with the shunt closed, by inspection. Two such typical curves are shown in Figure 4. When the shunt was open, the downslope was prolonged, the disappearance time prolonged, the peak concentration less and the least concentration and recirculation concentration greater. Without the control, however, none of the measurements shown in Tables I and II would separate the curve obtained with the shunt open from that with the shunt closed. Likewise, no ratios involving time or concentration could be found which increased the degree of separation between the two groups. The results for several such ratios are shown in Figures 5 and 6. The formulas used in the calculations are given in the figures except for the disappearance ratio. This is calculated from a concentration on the downslope at a time determined as the sum of build-up time plus peak concentration time, divided by the peak concentration.

Forward output did not correlate with any of the time measurements, Figure 7, ($r < 0.2$ for all times) so that using output in the ratios did not increase the separation between open and closed measurements. Finally, no equation such as that suggested by Korner and Schillingford (4, 5) using both volume and flow could be developed. Their formula was a regression equation for the reciprocal of the downslope using cardiac output and central blood volume. There was no correlation between slope and cardiac output either with the shunt open or closed ($r = 0.17$ closed and 0.29 open). Likewise, there was no correlation between slope and central blood volume ($r = 0.177$ closed and 0.125 open), so that no such regression equation could be developed. Actually, the only correlations were between central blood volume and cardiac output ($r = 0.869$ closed and 0.838 open), and between slope volume and cardiac output ($r = 0.884$ closed and 0.790 open) which are inherent in the calculations. Finally, although the curve obtained with the shunt open could be separated from that with the shunt closed, by inspection, the difference between the two curves did not correlate with the per cent regurgitation so that even with a control, no quantification was possible.

DISCUSSION

The effect of valvular incompetence on the dye dilution curve has been extensively studied both in models and patients by several groups including Korner and Schillingford in England (4, 5) and Keys, Woodward, Swan, Burchell and Wood (2, 3) at the Mayo Clinic. In theory, the effect of incompetence would be to delay the washout of the indicator from the heart chambers and this can be well shown in models. For any given flow and volume, incompetence will prolong the downslope and lower the peak concentration. In patients, likewise, the effect of mitral insufficiency on the dye curve has been shown to be a prolongation of

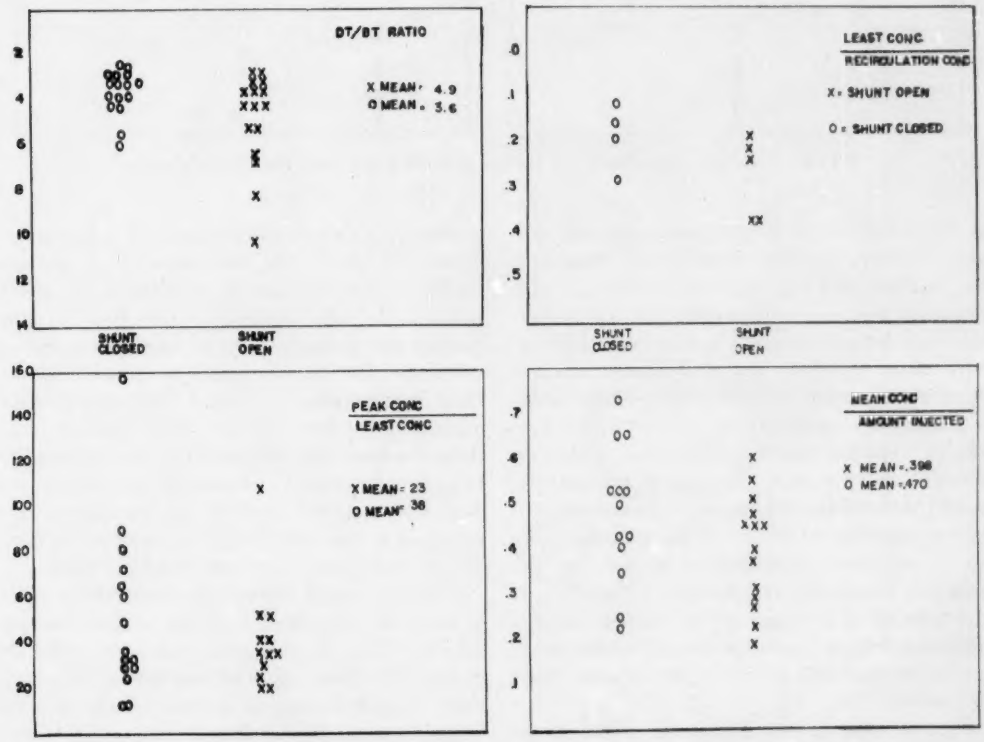

FIG. 5. TIME AND CONCENTRATION RATIOS DERIVED FROM DYE DILUTION CURVES

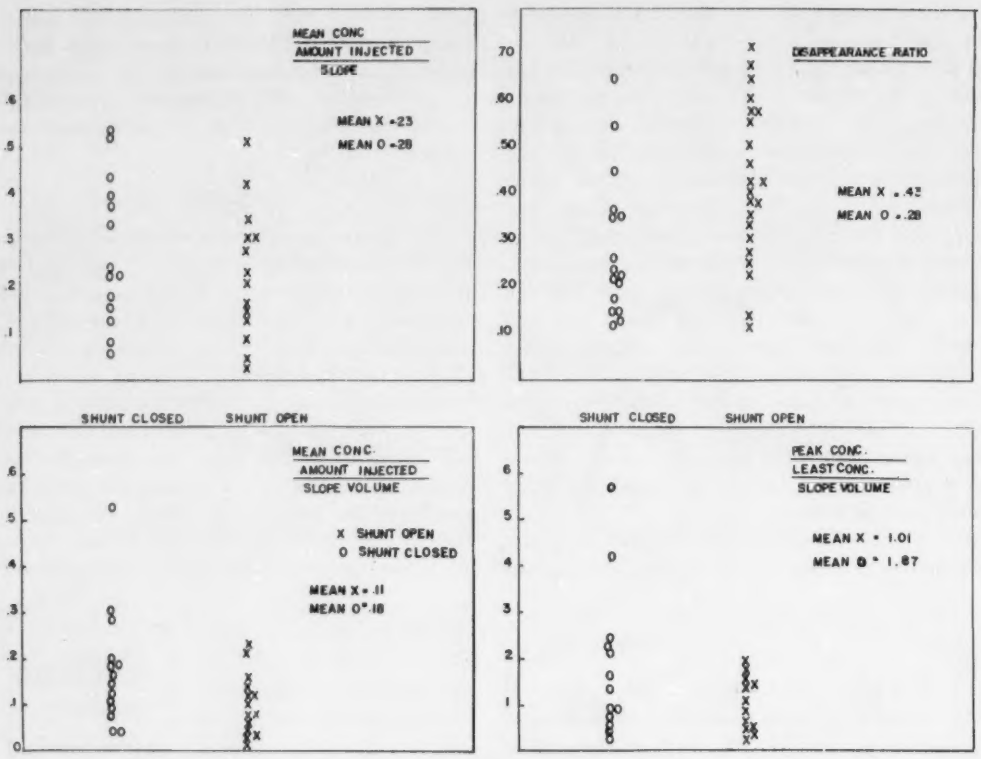

FIG. 6. TIME AND CONCENTRATION RATIOS DERIVED FROM DYE DILUTION CURVES

the downslope and an increase in the ratio between least concentration and recirculation concentration, without affecting appearance time or mean circulation time. From this work, various calculations have been developed which, when applied to a dye curve, have been used to determine whether mitral regurgitation is present and in some cases to quantify the regurgitation. However, the dangers of applying model dye dilution studies to patients are very real (9) and in the patients studied, an accurate measurement of the amount of regurgitation for calibration of the technique may not be obtained, depending as it does on the surgeon's impression at operation. Therefore, it was believed to be important to evaluate the dye dilution technique in an experimental animal preparation in which the pitfalls of model work could be avoided.

It is, of course, true that acute studies in an experimental animal are not completely comparable to a chronic disease such as mitral insufficiency in man. The size relationship between the left auricle and ventricle is different, the shunt is not in the same position in relation to the flow through the ventricle as in an incompetent mitral valve, and the hemodynamics are altered by the chest being opened combined with an extensive surgical procedure. It was felt, however, that these disadvantages were offset by the opportunity to accurately control and measure the regurgitant flow and to obtain control and regurgitant dye curves in a system in which the variables of flow, volume and mixing were not artificially fixed.

A shunt of small dimensions was chosen in order to keep the regurgitant flow low so that the usefulness of the dye dilution technique could be evaluated in detecting small amounts of regurgitation. With this shunt the per cent of regurgitation varied between 20 and 38 per cent, which is said not to be significant regurgitation in terms of

contraindication for mitral commissurotomy (1). This amount of regurgitation was sufficient, however, to elevate the left auricular pressure approximately 1 ml. Hg and on clamping the shunt, a transient rise in ventricular pressure was found which is similar to the finding of Wiggers and Feil (10).

Although most of the studies on the use of this dye dilution technique to measure mitral insufficiency have been done using a cuvette oximeter and a continuous recording system, the interrupted sample technique was chosen for this study. While the frequency response may not be comparable to a continuously recorded curve, it is a simple, well standardized technique to obtain a dye dilution curve (9). By this technique, satisfactory agreement between cardiac output using the pulmonary artery injection compared to left atrial injection was obtained, as well as satisfactory agreement between two left auricle injections done 10 minutes apart with the shunt open. Likewise, the effective cardiac output was the same with the shunt open or closed as measured by the dye technique, which has been shown to be true by Braunwald, Welch and Sarnoff, using a turbine flowmeter to measure output (11).

In spite of the small regurgitant flow chosen for the studies, the curve obtained with the shunt closed, which was used as a control, could always be separated from the curve taken with the shunt open by simple inspection of the two curves. The curve obtained with the shunt open always had a more prolonged downslope. Without a control, however, as can be seen by the wide range of values in Tables I and II, no separation could be obtained by slope or any other single measurement of time or concentration applied to the curve. It should be pointed out, however, that with one second sampling intervals, measurements of time components such as the build-up time, which

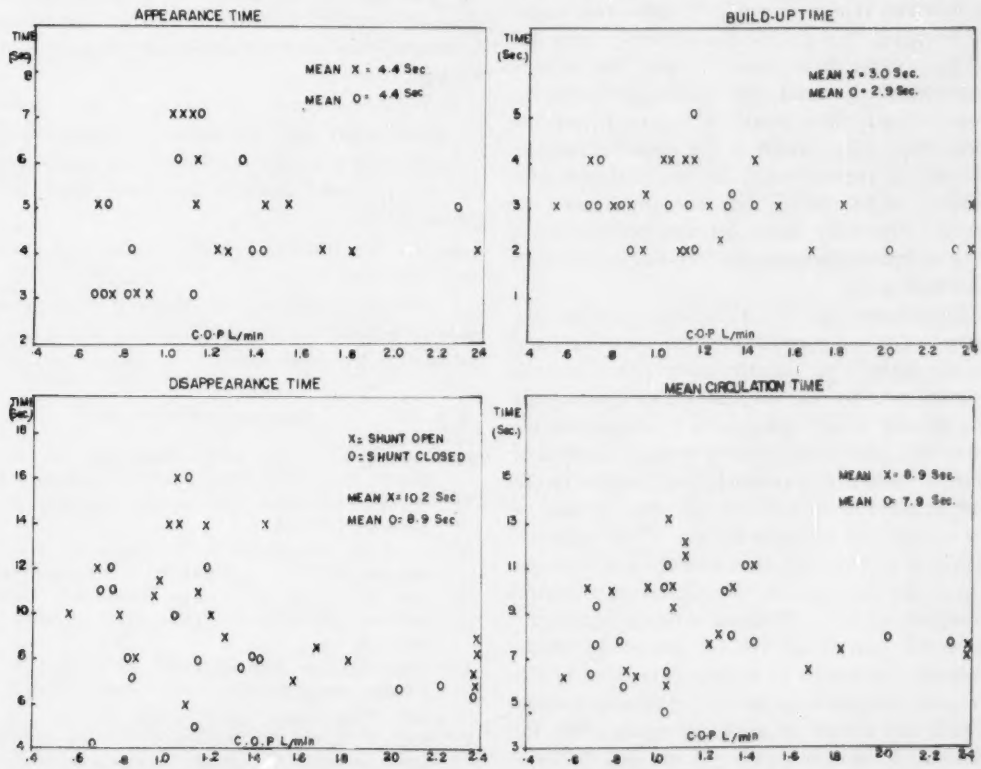

FIG. 7. THE RELATIONSHIP OF CARDIAC OUTPUT AND THE TIME COMPONENTS OF THE DYE CURVES

range from two to five seconds, are subject to large relative errors. Likewise, the measurement of specific points on the curve, such as the peak concentration, will be subject to error.

In theory, it might be expected that by combining two components of the curve which are altered in different directions by mitral regurgitation, a better separation could be obtained. For example, in regurgitation, the slope is prolonged so that the least concentration is increased while the recirculation concentration is decreased. The ratio of these two would thus be greater in mitral insufficiency than the changes in either one alone. Various ratios including the ratio of least to recirculation concentration were applied to the data as seen in Figures 5 and 6, but the wide overlap was not appreciably altered and without a control curve, no useful separation could be obtained.

This scatter between values obtained either with the shunt open or closed is not unexpected since the characteristics of the dye curve are dependent on flow and volume as well, and these were variable from dog to dog. No calculation could be developed, using flow alone or flow and volume together, which would separate the open from the closed shunt. This would be expected from the data, since slope, which is the major parameter affected by regurgitation, did not correlate with cardiac output, either with the shunt open or closed. The wide scatter between determinations likewise makes quantification impossible even with a control curve.

The explanation for the inability of the dye curve to detect regurgitation without a control and for the inability to quantify even with a control, would appear to rest with factors such as volume and mixing which influence the contour of the curve and which cannot be accurately assessed at present. First, it is probably not possible to obtain an accurate measure of the blood volume of the auricle and ventricle by use of the Stewart-Hamilton method (6). At best there is a 10 per cent methodical error in the calculation of central blood volume (12). Because of this, a meaningful correction for blood volume cannot be made. Secondly, variations in mixing either due to differences in injection or to the presence of laminar flow in the auricle or ventricle would affect the contour of the dye curve. It has been demonstrated by Woodward, Swan, Wood and Marshall (13, 14) that such incomplete mixing may occur in the left auricle or left ventricle. Likewise, it has been shown by Hoffman and Rowe (15) in models, that the nature of the regurgitant jet and the chamber into which it enters, affect the indicator dilution curve. At present, no meaningful correction for these variations can be applied to the curve.

In spite of the variabilities, the fact remains that with a control curve rather small amounts of regurgitation can be detected by any simple analysis. In this experimental preparation, therefore, the validity of the dye dilution technique to demonstrate the presence of regurgitation seems well established. Whether the technique can be refined to quantify regurgitation remains to be studied.

CONCLUSIONS

1. The usefulness of the dye dilution technique for detecting mitral insufficiency in the range of 20 to 38 per cent of cardiac output was evaluated by means of an acute experimental preparation in 15 dogs.

2. With a control, the technique was good enough to detect small amounts of regurgitation.

3. Without a control, no calculation applied to the curve would separate the open from the closed shunt.

4. No quantification could be obtained even with a control.

5. Reasons for this wide overlap rest with variations in volume, mixing and washout, which cannot be assessed at present.

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CHRONIC PROGRESSIVE MYOPATHY WITH MYOGLOBINURIA: DEMONSTRATION OF A GLYCOGENOLYTIC DEFECT IN THE MUSCLE *

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Although little is known of the metabolic disturbances in the various myopathies of man and animals (1), in 1956, one of us (R. S.) observed a patient with a bizarre muscle disorder, associated with paroxysmal myoglobinuria, whose unusual clinical manifestations suggested a possible lead as to the nature of the metabolic defect involved. In this patient, the most striking feature was his inability to perform moderately severe muscular work even over short periods of time, whereas muscular activity of a *minimal* degree was tolerated almost without limitations. For example, moderate exercise, such as climbing 10 steps of a stairway, produced severe and painful cramps in the involved muscle groups, lasting for approximately an hour, and was followed by transient myoglobinuria. Contrariwise, slow walking on level ground was tolerated for hours without developing cramps or even undue fatigue. Moreover, it was found that during and following moderate exercise, which was sufficient to produce a cramp, the expected rise in blood lactate concentration (2–4) did not occur. These observations suggested that the patient may suffer from a defect in glycogenolysis, limiting the energy available for muscular contraction to sources other than anaerobic breakdown of glycogen. This possibility was studied *in vivo* and *in vitro* (5).

CLINICAL DATA

Investigations on this patient were initiated in 1957 at the National Institutes of Health [1] and were continued in the summer of 1958 at the Thorndike Memorial Laboratory, Boston City Hospital.

The patient was a single, 52 year old male of Portuguese extraction, who had lived all his life in California. Since childhood he had become easily fatigued and had been unable to keep pace with other children. At the age of 20 he began to notice that following exercise, cramping pain and weakness developed in the muscles of his limbs; such episodes were usually followed by transient excretion of dark urine. For instance, stooping or crouching for a few seconds induced painful contractures of the muscles of his thighs and calves, the cramps lasting for one to two hours. After gradual relaxation, the involved muscle groups exhibited a putty-like consistency and extreme weakness which subsided slowly over the next two weeks. Following such an acute episode, the urine regularly turned dark brown, but usually reverted to a normal color within 12 hours.

These manifestations, which occurred with varying degrees of severity, were most pronounced in the proximal muscles of the four extremities, but at times involved the calves and the strap muscles of the back. His disability forced him to take a job as a doorman, in which occupation little demand was made on his physical performance. Over the years he observed a slow loss of strength in his arms and legs, and in 1947 he was admitted to the University of California Hospital for study and possible treatment.

A report of these investigations (6) revealed no obvious abnormalities, except for weakness of the upper part of the arms and thighs together with slight wasting of the proximal limb muscles, which suggested the diagnosis of progressive muscular dystrophy. The dark urinary pigment, excreted after episodes of cramps, was identified as myoglobin; in addition, such urine specimens contained large amounts of creatine. A series of other laboratory tests was performed, the results of which were believed to be essentially normal. On re-examination of the published data (6), however, it is apparent that following exercise the concentration of lactic acid in the blood showed a distinct decrease, instead of the expected rise. The significance of this finding was not appreciated at that time.

Since this report in 1948 (6), the patient's condition has slowly deteriorated. His musculature has become progressively weaker and atrophic, involving particularly the thighs, the upper arms and the shoulder girdle. Se-

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vere attacks of cramps and myoglobinuria have occurred less frequently. This may, in part, have been due to the patient's having learned to avoid situations prone to induce such episodes. In spite of his semi-invalid condition, he stayed on his job until summer 1957, when because of a fall and injury in descending a stairway, he was forced to retire.

Since that time, he has been taking daily walks of 10 to 20 blocks on level ground at slow pace to keep up his physical exercise. He has great difficulty, however, in entering or leaving a bus or taxicab, or climbing even a few steps of a stairway. Over the past year he has noticed the onset of difficulties in chewing, resulting from cramplike pain in his muscles of mastication.

His past history included pulmonary tuberculosis at age 20, gonorrhea at 27, and syphilis at 30. At the time of the present studies, repeated X-ray and laboratory tests failed to reveal any evidence for continued activity of these conditions.

The patient has nine living siblings, none of whom has noticed abnormalities in muscle function or excretion of dark urine. One sister died at the age of 20 of pulmonary tuberculosis. His father died at 80 from carcinoma of the lung, while his mother is still living and well at 82.

For all of his adult life the patient has smoked approximately one package of cigarettes per day and has taken alcohol only sparingly. He denies the use of drugs, or known exposure to toxins.

Physical examination revealed a temperature of 98.8° F., blood pressure 124/96, regular pulse rate of 76 and respiratory rate of 18. He was a pyknic, slightly obese white male in no apparent distress (Figure 1). Abnormal physical findings were limited to the muscular system. There was general wasting of the proximal muscles of the extremities and the shoulder girdle. In the upper limbs the muscle groups most severely affected included biceps, triceps, infraspinatus, pectoralis, trapezius and latissimus dorsi, as well as the sternomastoids. The muscles of the forearms and the hands were fairly well preserved and so were the muscles of the trunk and spine. In the lower extremities, atrophy and weakness were most severe in the quadriceps and adductors, but the calves appeared to be actually hypertrophic. No atrophy was noticed in the muscles supplied by cranial nerves. There was no percussion myotonia or fasciculation. The tendon reflexes were equal on both sides, but were quite hypoactive. Pathological reflexes were absent. The cranial nerves and the sensory system were intact.

X-ray examinations of the chest and the gastrointestinal tract were normal. The heart was not enlarged and two electrocardiograms failed to reveal significant abnormalities.

The following laboratory examinations, all giving results within the normal range, were performed:

Blood. Hemoglobin level was 15.6 Gm. per cent; RBC, 5.6 millions; hematocrit, 50 per cent; platelets, 370,000; leukocytes, 6,300; granulocytes, 61 per cent; lymphocytes, 28 per cent; monocytes, 9 per cent; eosinophiles, 2 per cent; Na, 138 mEq.; K, 4.0 mEq.; CO_2, 31 mEq.; Cl, 104 mEq.; BUN, 10 mg. per cent, creatinine, 0.7 mg. per cent; fasting blood sugar, 72 mg. per cent; total protein, 6.3 Gm. per cent; globulin, 3.0 Gm. per cent (7); albumin, 3.3 Gm. per cent; bilirubin, 0.3 mg. per cent (8); serum iron, 96 μg. per cent; total serum iron-binding capacity, 124 μg. per cent; protein-bound iodine, 5 μg. per cent; prothrombin time, 11 seconds (control 11 seconds); cephalin flocculation negative; alkaline phosphatase, 1.9 Bessey-Lowry units; Kolmer, negative; VDRL, less than 1 unit.

FIG. 1. APPEARANCE OF THE PATIENT IN SUMMER OF 1958, DEMONSTRATING THE MUSCULAR WASTING IN THE PROXIMAL PARTS OF THE EXTREMITIES AND THE SHOULDER GIRDLE

The calves show muscular hypertrophy.

Urine. The urinary pH was 4.8 to 6.8; concentration was 1.001 to 1.032; reducing substance, protein and bile pigments were absent; formed elements were absent; xanthurenic acid (9) was not demonstrable; 5-hydroxyindolacetic acid (10) excretion was 6.0 mg. per 24 hours; PSP excretion was 44 per cent in 15 minutes and 61 per cent in 30 minutes; myoglobin (11, 12) was absent before exercise, but present in small amounts after exercise.

Feces. The feces were negative for blood; urobilinogen (13) excretion was 71.6 mg. per 24 hours.

Radioactive iodine uptake was 24.8 per cent in 24 hours.

TABLE I

Effect of epinephrine and of glucagon on blood sugar, lactate, pyruvate, NEFA, phosphorus and potassium*

Time	Glucose	Lactate	Pyruvate	P	K	Glucose	Lactate	Pyruvate	P	K	NEFA*
min.	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mEq./L.*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mEq./L.*	*μEq./L.*
Control	65	4.4	1.4	2.9	4.6	78	5.6	1.2	2.8	4.2	740
0	0.5 mg. Epinephrine, (s.c.)					Glucagon, 1 mg. (i.v.)					
10						125	5.6	1.2	1.7	4.3	745
15	77	6.0	1.4	2.7	4.4						
20						123	7.0	1.4	1.6	3.8	690
30	91	7.2	1.3			113	7.0	1.4	1.8	3.4	515
45	108	8.0	1.3			155	6.8	1.4	1.6	3.5	375
60	113	7.2	1.3	2.7	3.7	116	7.4	1.4		3.9	340
90	105	8.4	1.4			91	7.6	1.5	2.5	3.8	290
120	91	7.4	1.4								

* Nonesterified fatty acids.

METHODS

All studies *in vivo* were performed with the patient and the control under fasting and resting condition. A healthy, 36 year old physician served as the control subject. For the oral glucose tolerance test, the patient was given 100 ml. of a 50 per cent glucose solution and the blood concentrations of glucose (14), lactate (15), pyruvate (16), phosphorus (17) and potassium (18) were determined at intervals of 30 minutes for the following three hours.

Epinephrine was injected subcutaneously in a dose of 0.5 ml. of a 1:1000 solution and glucagon intravenously in a dose of 1 mg. (Table I). Nonesterified fatty acids (NEFA) were determined by the method of Dole (19).

The combined glucose and insulin test (Table II) was carried out as follows. The fasting patient was given a rapid intravenous injection of 11 Gm. of glucose in water and was then connected with a Bowman constant infusion pump which delivered 20 Gm. of glucose in water per hour. After 30 minutes on the pump, 10 units glucagon-free insulin [2] was rapidly injected through a separate vein and blood samples were withdrawn at the time indicated (Table II).

TABLE II

*Effect of intravenous glucose and insulin on blood glucose, lactate, pyruvate, phosphorus and NEFA**

Time	Glucose	Lactate	Pyruvate	P	NEFA*
min.	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*μEq./L.*
Control	69	4.6	1.2	2.8	940
0	Glucose, i.v. (11 Gm. during first 5 min., then 20 Gm./hr. at constant rate)				
30	170	7.2	1.1	1.9	605
35	(10 Units glucagon-free insulin, i.v.)				
45	161	9.5	1.4	2.0	515
55	128	9.9	1.2	1.8	350
65	120	9.5	1.5	1.5	370
75	109	10.0	1.4	1.5	320
95	111	11.9	1.5	1.7	330

* Nonesterified fatty acids.

[2] Obtained from Eli Lilly Co., through courtesy of Dr. W. R. Kirtley.

For the exercise tolerance test, the patient walked on a treadmill at an angle of 2° and a speed of two miles per hour, and the time which elapsed until cramps in the legs occurred was determined. After a rest of 30 minutes, the experiment was repeated with the patient connected to the Bowman pump, which delivered 0.9 per cent saline at a constant rate of 200 ml. per hour. In order to minimize subjective factors, the patient was made to believe that he was getting glucose and insulin

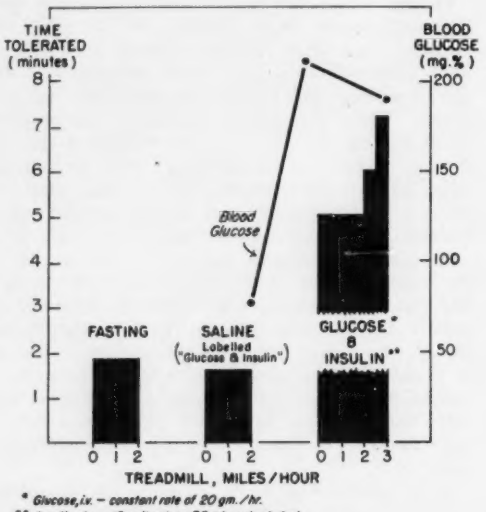

FIG. 2. EXERCISE TOLERANCE (TREADMILL) IN FASTING STATE, WITH SALINE INFUSION AND WITH INFUSION OF GLUCOSE AND INSULIN

The treadmill was inclined at an angle of 2°. Exercise was terminated when cramps developed (fasting and saline) or when the patient became dyspneic (glucose and insulin).

TABLE III

Plasma and urinary findings before and after exercise

	Venous plasma				Urine, 24 hr. collection				
	pH	K	O_2	CO_2	Volume	Creatine	Creatinine	K	Myoglobin
		mEq./L.	*vol. %*	*vol. %*	*ml.*	*mg.*	*mg.*	*mEq.*	
Before exercise	7.40	3.9	11.8	51.1	1,020	460	768	73	Absent
5 Min. after exercise*	7.38	3.8	6.8	52.6					
After exercise					2,470	776	970	161	Present

* Exercise consisted in assuming a crouched position for 20 seconds, which resulted in severe cramps in both thighs and calves.

instead of saline. After a second rest period of 30 minutes, he was given a rapid infusion of 11 Gm. of glucose, followed by constant infusion of glucose at a rate of 20 Gm. per hour. Thirty minutes before the third exercise test, an intravenous injection of 10 units glucagon-free insulin was given. During the test, the speed of the treadmill was increased to 2.5 and finally to 3.0 miles per hour (Figure 2).

Other exercise tests were performed as follows: with the patient at rest, urine was collected over a 24 hour period for quantitative determination of creatine, creatinine and potassium excretion (18, 20) as well as for the demonstration of myoglobin (11, 12). The patient was then asked to assume a crouching position for 20 seconds, at which time severe cramps developed in both thighs and calves. Venous blood specimens were drawn from the femoral vein before and five minutes after the excercise for estimation of pH, CO_2, O_2 and potassium, and the urine was collected for 24 hours following the exercise test (Table III).

Another type of exercise test was performed by having the patient climb briskly 20 steps of a stairway. This resulted in severe cramps in the legs, making it necessary to carry the patient to his bed. Blood samples were withdrawn before and at various time intervals after exercise for determination of lactate, pyruvate and NEFA.

Ischemic exercise in the forearm was studied by the following method. After a rest period of 30 minutes, a cuff inflated to 200 mm. Hg was applied around the wrist to prevent admixture of arterial blood from the hand, and venous blood was then withdrawn without

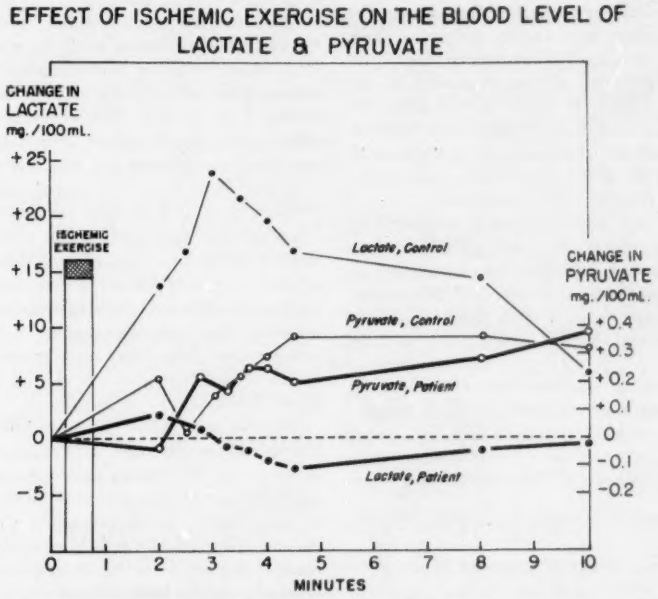

FIG. 3. THE ISCHEMIC EXERCISE CONSISTED IN PUMPING WATER WITH A SPHYGMOMANOMETER BULB WHILE ARTERIAL AND VENOUS BLOOD FLOW WERE OCCLUDED

TABLE IV

Lactate produced by muscle homogenate without added substrate and with addition of glycogen or phosphorylase in vitro

Source of tissue	Addition	Lactate formed	
		μMoles/ Gm.	*μMoles/mg. protein-N*
Patient, trapezius		2.2	0.06
Patient, trapezius, same	epinephrine*	2.0	0.06
Patient, trapezius, same	glycogen R†	1.9	0.05
Patient, trapezius, same	phosphorylase‡	6.8	0.19
Patient, lat. dorsi		2.8	0.07
Patient, lat. dorsi, same	epinephrine*	3.4	0.08
Patient, lat. dorsi, same	glycogen R†	2.9	0.07
Patient, lat. dorsi, same	glycogen H†	2.9	0.07
Control, quadriceps		13.0	0.32
Control, quadriceps, same	phosphorylase‡	15.0	0.37
Control, sartorius		9.5	0.24
Control, rectus abd.		13.2	0.30
Control, quadriceps		11.7	0.29
Control, quadriceps (3 weeks immobilized)		8.4	0.20

* Tissue slices preincubated with epinephrine for 10 minutes prior to preparation of homogenate.

† Addition of 3 mg. glycogen prepared from human (H) or rat (R) muscle.

‡ Addition of 2 mg. crystalline animal phosphorylase.

stasis from the antecubital vein. A second cuff around the upper arm was then inflated to 200 mm. Hg and the muscles of the forearm were worked ischemically for 45 seconds by pumping water against a resistance with a sphygmomanometer bulb. One minute after the end of the ischemic exercise, the cuff around the upper arm was deflated and blood samples were rapidly withdrawn from the antecubital vein (Figure 3). A healthy individual served as control, performing the same amount of exercise over the same length of time. In the latter, the exercise was accomplished without undue fatigue, whereas the patient developed severe cramping pain in the muscles of the ischemic forearm and hand. In both experimental subjects the test was repeated on a separate day.

Blood flow before and after exercise was studied by occlusion plethysmography of the whole leg (21).[3]

The glycogen content of leukocytes was determined by the method of Seifter, Dayton, Novie and Muntwyler (22).[4] The rate of glycolysis of shed blood on aerobic incubation was estimated by the method described by Bird (23).

For incubation, muscle specimens were obtained by biopsy of various muscle groups under local or general anesthesia.[5] The anesthesia employed did not appear to interfere with the assay of the enzyme systems studied. Five individual biopsies of different muscles were performed on the patient and five biopsy specimens were obtained from control subjects who did not suffer from musculo-skeletal diseases. In addition, one biopsy was performed on the quadriceps of a 70 year old woman who had been totally immoblized for the preceeding three weeks because of a comminuted fracture of the hip. The tissue specimens were immediately put into ice cold 1.5 per cent KCl and processed at once.

For estimation of anaerobic glycolysis, approximately 1 Gm. of muscle was blotted dry, weighed on a torsion balance and thoroughly homogenized with four volumes of ice cold 0.3 M Tris (hydroxymethyl aminomethane) buffer, pH 7.5, containing 4×10^{-3} M $MgCl_2$. A loose-fitting hand homogenizer was used and the homogenate was spun for 1 minute at 200 rpm to remove large particles of connective tissue. All operations were carried out in the cold.

For incubation, the mixture contained 0.5 ml. homogenate, 1×10^{-3} M adenosine triphosphate (ATP), 5×10^{-4} M diphosphopyridine nucleotide (DPN), 4×10^{-2} M nicotinamide, 1×10^{-2} M cysteine, substrate where applicable and Tris buffer to make a final volume of 2 ml. In those experiments where substrate was added, 20 μMoles glucose-1-phosphate, glucose-6-phosphate, or fructose-1, 6-diphosphate or 3 mg. of human or animal glycogen was present per flask. In some instances, 2 mg. muscle phosphorylase[6] in microcrystalline suspension was added.

Incubations were carried out in an atmosphere of 95 per cent N_2, 5 per cent CO_2 for thirty minutes at 38° C. in a Dubonoff shaking water bath. At the end of the incubation, the reaction was terminated by the addition of 3 ml. 10 per cent trichloracetic acid. In some instances (Table IV), muscle slices of approximately 100 mg. were preincubated for 10 minutes at 38° C. with Tris buffer, containing 2 μg. per ml. of epinephrine to assure complete conversion of phosphorylase B to A (24–26). The tissue was then homogenized and incubated as indicated above. Glycogen was freshly prepared from human muscle obtained at surgery or from rat muscle (27).

Lactate was estimated by the method of Barker and Summerson (15), all assays being done in duplicate. The amount of lactate formed during incubation was calculated as the difference between lactate concentration in the incubated flask and that present in the original muscle homogenate. The latter was measured by adding 0.5 ml. of chilled homogenate to 4.5 ml. of 10 per cent trichloracetic acid.

Phosphorylase activity was estimated by the method of Sutherland and Wosilait (25) using glucose-1-phosphate as substrate. The results were expressed in milligrams of inorganic phosphorus liberated in 30 minutes per gram of tissue used. In some instances (Table V), the reaction mixture contained 0.02 M adenylic acid or 1×10^{-6} M epinephrine, or prior to homogenization, tissue slices of approximately 100 mg. were preincubated for 10 min-

[3] This determination was kindly performed by Dr. W. H. Abelmann.

[4] This determination was kindly performed by Dr. Jane Shohl.

[5] The biopsies were kindly performed by the staff of the Fifth (Harvard) Surgical Service of the Boston City Hospital.

[6] Sigma Co., St. Louis, Mo.; Lot No. P38-98, 1,200 units per mg.

utes at 38° C., in 0.1 M NaF, containing 2 μg. per ml. of epinephrine.

Muscle glycogen was determined by the method of Good, Kramer and Somogyi (28) and expressed in per cent of muscle wet weight. Protein nitrogen was determined by the micro-Kjeldahl method (29).

For histological examination, muscle tissue was fixed in Zenker's solution and sections were stained with hematoxylin and eosin, periodic acid-Schiff or with Best-carmine. In some experiments, Zenker-fixed tissue sections were washed and then incubated for three hours at 38° C. in 0.9 per cent saline containing 50 mg. per ml. animal diastase.[7] Similar sections cut from the same paraffin block were incubated with heat-inactivated enzyme. At the end of the incubation period the tissues were washed, stained with Best-carmine and the amount of red staining material was estimated.

RESULTS

A. In vivo studies of carbohydrate metabolism in the resting patient

The oral glucose tolerance test revealed a normal response (30), with blood glucose concentrations rising from a fasting level of 81 mg. per cent to 140 mg. per cent in 30 minutes and returning to 65 mg. per cent after 90 minutes. Concomitantly, there was a transient rise in blood lactate concentration, the respective values being 7.2 mg. per cent fasting, 11.6 mg. per cent after 30 minutes and 7.2 mg. per cent after 90 minutes. Changes in phosphorus and potassium concentration were insignificant.

The data obtained after administration of epinephrine or glucagon are summarized in Table I. Both compounds produced a transient hyperglycemia, which was comparable in degree to that seen in normal individuals (31, 32), suggesting adequate glucose mobilization in the liver. There was a simultaneous rise in lactate concentration, whereas pyruvate remained essentially unchanged. Phosphorus levels were low in the fasting state and both epinephrine and particularly glucagon produced a further fall to values which were distinctly below normal (32, 33). The decrease in potassium concentration was similar to that found in normal individuals (34). Glucagon administration was followed by the expected fall in NEFA concentration (35).

The effect of intravenous injection of glucose and insulin on the level of lactate, pyruvate, phosphorus and NEFA is summarized in Table II. Administration of glucose alone produced an increase in lactate and a fall in NEFA concentration; the addition of insulin further enhanced this effect (9, 31, 36–38). Similar to the findings in Table I, the hyperglycemia was accompanied by a low phosphorus level and the values were further depressed after the administration of insulin (33, 39).

B. In vivo studies before and after exercise

The results obtained in the exercise test which involved climbing 20 steps of a stairway are given in Figure 4. In the control subject there was the expected rise in lactate, pyruvate and NEFA concentrations (2–4, 40), with pyruvate increasing from 1.05 mg. per cent to 1.9 mg. per cent six minutes after exercise. Contrariwise, in the patient, lactate and pyruvate did not increase, but remained at or slightly below the control values obtained at the onset of the experiment. For pyruvate, the fasting level was 1.32 mg. per cent and the values 1, 5, 15 and 35 minutes after exercise were 0.92, 1.18, 1.06 and 1.16 mg. per cent, respectively. In contrast to these results, the rise in NEFA concentration was com-

TABLE V

Phosphorylase activity in muscle homogenate

Source of tissue	Addition	P_{in}* liberated
		mg./Gm.
Patient, trapezius		0.1 †
Patient, lat. dorsi		1.9
Patient, gastrocnemius		0
Patient, gastrocnemius, same	epinephrine, 1×10^{-6} M	0
Patient, gastrocnemius, same	epinephrine‡	0
Patient, gastrocnemius, same	AMP, 0.02 M§	0
Control, quadriceps		54
Control, sartorius		58
Control, rectus abd.		17
Control, rectus abd.		25
Control, rectus abd., same	epinephrine‡	42
Control, quadriceps (3 weeks immobilized)		1.9
Control, quadriceps (3 weeks immobilized), same	AMP, 0.02 M§	13
Control, rectus abd.		38
Equal parts of above and patient's muscle		18

* P_{in} = inorganic phosphorus.
† Average of duplicate determinations.
‡ Tissue slices preincubated with epinephrine for 10 minutes prior to preparation of homogenate.
§ AMP = adenylic acid.

[7] Nutritional Biochemicals Corporation.

parable to that found in the control subject. Blood glucose concentration did not change significantly in either of the experimental subjects.

In the ischemic exercise test (Figure 3), the difference between patient and control subject was even more striking. Here, the return of blood from the exercised limb to the liver was obstructed, permitting a closer evaluation of the metabolic events during muscular contraction. As seen in Figure 3, ischemic exercise of the control subject resulted in a prompt and manifold increase in lactate concentration, with a lesser rise in pyruvate. In the patient, on the other hand, there was a minimal increase in lactate concentration during the initial postexercise period, with a subsequent fall below the control values which were obtained prior to the exercise. The initial small rise, observed immediately after releasing the pressure cuff around the upper arm, probably reflected stasis of blood (41), rather than lactate production in the exercised muscle. In contrast to the values for lactate, pyruvate concentration showed a slight increase, similar in extent to that observed in the control subject. In neither of the two experimental subjects was there a significant change in potassium concentration.

Ischemic exercise tests were also performed in five other patients with neuromuscular disorders, including three patients with familial muscular dystrophy, and one patient each with amyotrophic lateral sclerosis and with myositis. In all instances, the results were similar to those obtained in the normal subjects, in that after exercise, lactate concentrations rose from 18.7 to 63.1 mg. per cent over the respective control values.

The plethysmographic determination of blood flow showed a mean resting blood flow of 0.5 ml. per 100 ml. leg volume per minute, which rose to 1.2 ml. during exercise. These values are within normal limits (21). Unfortunately, the status of the patient's peripheral vessels in all four extremities did not permit more extensive studies of blood flow at rest or during exercise.

As seen in Table III, exercise resulted in a slight decrease in venous pH and in increased oxygen extraction by the exercised limb. Carbon dioxide and potassium concentrations did not show significant changes. Urinary excretion of creatinine was decreased, while excretion of creatine was greatly increased at rest (1, 30), and may have risen further after exercise. Many attempts to demonstrate myoglobin in urine at rest were unsuccessful, but after exercise, small amounts of myoglobin could at times be identified. It should be noted, however, that after exercise, the urine did not change color and the amounts of myoglobin present appeared to be very much smaller than those found in a previous study of this patient reported in 1948 (6).

C. Studies of muscle tissue in vitro

In view of the finding that, on exercise, the patient's muscles failed to produce lactic acid, anaerobic glycolysis of muscle tissue was studied *in vitro*. In preliminary experiments, it was observed that in spite of a high glycogen content, incubation of muscle homogenates, *without* added substrate, produced little lactate, whereas with fructose-1, 6-diphosphate, glucose-6-phosphate or glucose-1-phosphate as substrates, lactate formation proceeded at a normal rate. This indicated the presence of a defect in glycolysis involving, most likely, the conversion of glycogen to glucose-1-phosphate. In subsequent experiments, these enzymatic steps were studied in more detail.

The glycogen content of the patient's muscles was found to be greatly increased (42), ranging from 2.4 to 3.0 per cent wet weight (three separate determinations). In spite of this, the amount of lactate formed on incubation was approximately 25 per cent of that, produced by similar preparations of normal muscles (Table IV). Addition of human or rat muscle glycogen to the patient's homogenate or preincubation of the tissue with epinephrine failed to increase lactate formation. On the other hand, addition of crystalline phosphorylase resulted in a more than threefold increase in the amount of lactate produced.

Among the homogenates prepared from control individuals, the least amount of lactate was formed by the muscle, which had been totally immobilized for three weeks (Table IV). Even in this preparation, however, glycolysis was significantly higher than in any of the patient's muscles. Addition of phosphorylase to homogenate of control muscle resulted only in an insignificant increase in lactate production. In contrast to these results, lactate formation with glucose-1-phosphate as substrate was the same in the patient's muscles and in

homogenates prepared from control individuals (Table VI). The lowest value was obtained with the immobilized muscle.

These findings indicated that the defect in glycogenolysis probably involved the phosphorylase system. Determination of phosphorylase activity in the patient's muscles revealed the absence of significant enzymatic activity (Table V). Addition of epinephrine (26, 43) or adenylic acid (25, 26) to the incubation mixture or preincubation of tissue with epinephrine failed to activate the enzyme system. On the other hand, muscle preparations obtained from control individuals showed phosphorylase activity comparable in magnitude to that reported in earlier studies (44). Preincubation of normal muscle with epinephrine or addition of adenylic acid to the homogenate, increased the enzymatic activity. This was particularly striking in the normal muscle, which had been immobilized for three weeks prior to biopsy.

The glycogen content of circulating leukocytes was found to be 71 and 72 mg. per 10^{10} cells, which is within the normal range (45), but differs from the increased value found in a case of hepatic glycogen storage disease (46). Incubation of shed blood revealed a normal rate of glycolysis (47).

TABLE VI

Lactate produced by muscle homogenate from glucose-1-phosphate in vitro

Source of tissue	Lactate formed	
	μMoles/ Gm.	*μMoles/mg. protein-N*
Patient, trapezius	55	1.5
Patient, lat. dorsi	35	0.9
Control, quadriceps	43	1.1
Control, sartorius	40	1.0
Control, rectus abd.	44	0.9
Control, rectus abd.	51	1.0
Control, quadriceps (3 weeks immobilized)	38	0.8

D. Exercise tolerance studies with infusion of glucose and insulin

The findings *in vivo* and *in vitro* indicated a muscular defect in glycogenolysis, making glycogen unavailable as a source of energy for muscular contraction. On the other hand, the effect of administration of glucagon, epinephrine and particularly of glucose and insulin in lowering the blood level of phosphorus and NEFA and increasing the lactate concentration suggested that muscular utilization of glucose may not be impaired. In support of this concept, it was noted that during and after infusion of glucose and in-

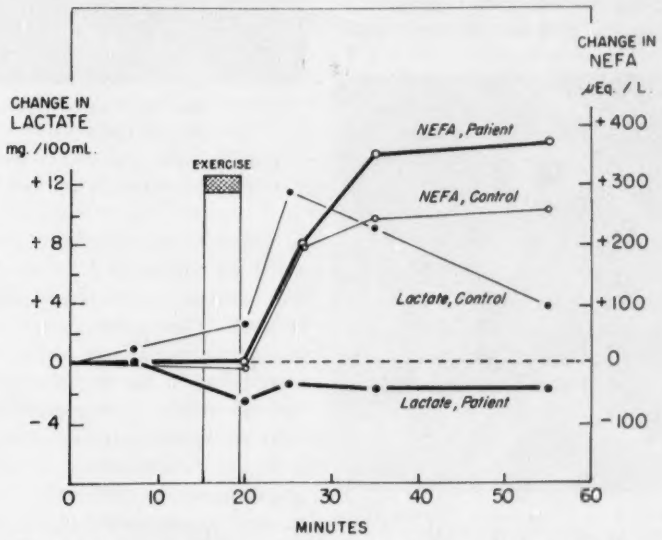

FIG. 4. EFFECT OF EXERCISE ON THE BLOOD LEVEL OF LACTATE AND OF NONESTERIFIED FATTY ACIDS (NEFA)

The exercise consisted in climbing briskly 20 steps of a stairway.

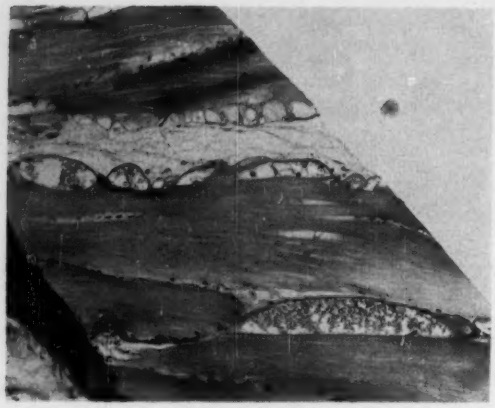

FIG. 5. LONGITUDINAL SECTION OF GASTROCNEMIUS STAINED WITH BEST-CARMINE

Hypertrophied muscle fibers and blebs of raised sarcolemma, containing granular glycogen, can be recognized.

sulin, the patient exhibited a noticeable increase in muscular strength. An attempt was made to quantify this improvement on the treadmill (Figure 2).

In the fasting state and during infusion of saline, the exercise test had to be terminated after a short period of time because of the development of cramps in the lower extremities (Figure 2). Contrariwise, during the infusion of glucose and insulin, the patient was able to walk for a much longer period and at increased speed, without the occurrence of cramps. After approximately seven minutes on the treadmill, the experiment had to be interrupted because of increased dyspnea, but the patient was able to resume walking after a short period of rest.

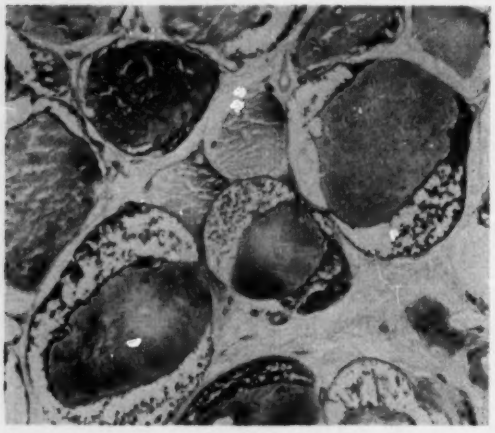

FIG. 6. CROSS SECTION OF QUADRICEPS STAINED WITH PARA-AMINOSALICYLIC ACID (PAS)

Large amounts of PAS-positive glycogen are present under the raised sarcolemma and within the muscle fibers (courtesy Dr. Thomas, National Institutes of Health).

E. Histological studies of muscle

The histological appearance of the muscle is illustrated in Figures 5 through 7. All muscle specimens were found to contain large amounts of a granular material which stained red with para-aminosalicylic acid (PAS) and Best-carmine. This material disappeared completely after incubating the tissue sections with diastase, whereas

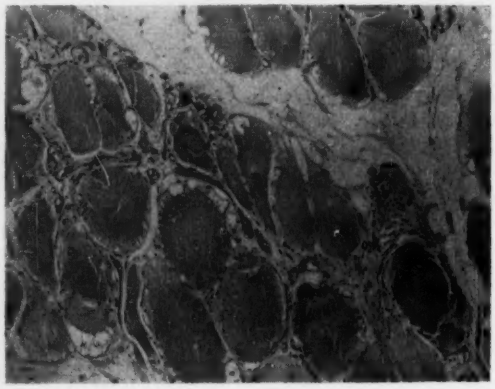

FIG. 7. CROSS SECTION OF GASTROCNEMIUS STAINED WITH HEMATOXYLIN AND EOSIN

A necrotic fiber is present on the right and a remnant of another fiber is seen in the upper left corner.

in specimens incubated with heat-inactivated enzyme, the amount of PAS and Best-carmine-positive material was similar to that found in control section. These observations indicated that the muscle contained large amounts of glycogen. In addition some sections showed muscle fibers in various states of degeneration (Figure 7) or scattered fibers exhibiting acute necrosis.

Figure 5 illustrates a longitudinal section of gastrocnemius stained with Best-carmine demonstrating hypertrophied fibers with internal chains of nuclei, and with blebs of raised sarcolemma. The latter contained granular material which stained bright red with the Best-carmine method.

In the upper two fibers, the granular material has partly disappeared and histiocytic nuclei are present within the blebs. Figure 6 is a cross section of quadriceps, stained with PAS. There is a similar accumulation of PAS-positive material under the raised sarcolemma and, in some fibers, infiltrating diffusely the sarcoplasm. Figure 7 is a cross section of gastrocnemius stained with hematoxylin and eosin. The granular material was largely washed out by the staining procedure. On the right is a necrotic fiber with histiocytic invasion and on the upper left a remnant of another damaged fiber.

DISCUSSION

Current physiological concepts indicate that adenosine triphosphate (ATP) plays a central role as the immediate source of energy which is required for muscular contraction as well as for the basic metabolism of muscle at rest (1, 42, 48). In resting muscle, the relatively small amount of high energy phosphate needed is largely generated through oxidation of noncarbohydrate materials (49), while oxidation of glucose to CO_2 and water, and anaerobic dissimilation of glucose to lactate appear to be of minor importance. However, even resting muscle which actively takes up O_2 produces a small amount of lactate which is almost all derived from degradation of glucose (49). Approximately 60 per cent of the glucose uptake by forearm muscles can be accounted for by lactate production (49), while anaerobic breakdown of glycogen appears to be of little importance as a source of energy at rest.

In contracting muscle, on the other hand, the increased energy requirements cannot be met by lipid and carbohydrate nutrients which diffuse from the blood into the muscle cells, nor is the oxygen supply adequate to sustain a predominantly aerobic oxidation (1, 42). Maximal activity of frog muscle, for instance, increases the rate of ATP utilization almost 1,000-fold above the resting rate (42). The energy for this muscular work must be largely supplied through anaerobic mechanisms and from substrates which are present within the muscle cells.

The amounts of ATP and of phosphocreatine stored in the muscle are insufficient to sustain anaerobic contraction for more than a very short period of time (42). For continued activity, the energy demand must be met by anaerobic glycolysis of glycogen to lactic acid. The latter diffuses into the interstitial fluid and is carried by the circulation to the liver, where it is in part oxidized to CO_2 and water and in part utilized for resynthesis of glycogen (42). The rise in lactate concentration, which is always observed after exercise, is a reflection of the greatly increased rate of anaerobic glycolysis in contracting muscles (2, 3).

In the patient studied, a moderate degree of exercise for a very short period of time resulted in severe cramps which prevented further use of the involved muscle groups for several days. Associated with this process was histological evidence of muscle necrosis (Figure 7) and transient myoglobinuria. These observations suggested that the patient may be unable to mobilize glycogen for contraction of his muscles and may depend solely on whatever other fuels the muscles are able to abstract from the blood. This concept was borne out by both the findings *in vivo* and the studies of muscle metabolism *in vitro*.

In vivo, exercise and ischemic exercise failed to result in increased lactate concentration in venous blood (Figures 2 and 3), whereas in the normal individual and in five patients with other forms of myopathies, comparable amounts of exercise resulted in marked elevation of venous lactate levels. The finding that exercising the patient's muscle under ischemic conditions did not lead to a rise in venous lactate concentration indicated that the lack of lactate response was independent of changes in muscular blood flow. *In vitro*, lactate production by the patient's muscle was greatly reduced in spite of the presence of increased amounts of glycogen. This defect was not corrected by preincubation of muscle tissue with epinephrine (26, 50), nor by addition of glycogen prepared from normal human muscle or from rat muscle. On the other hand, addition of crystalline phosphorylase greatly increased lactate formation (Table IV). This finding and the demonstrated absence of phosphorylase activity, even in the presence of epinephrine or adenylic acid (26, 50) (Table V), indicated that the primary defect involved the phosphorylase system.

In normal muscle that has been exercised (26, 50) or totally immobilized (Table V), phosphorylase may be largely present in its metabolically

inactive form "b," but conversion to the enzymatically active form "a" can be achieved by epinephrine (26, 50). This is illustrated in Table V where preincubation of normal rectus muscle with epinephrine resulted in significantly increased phosphorylase activity. Moreover, addition of adenylic acid to homogenate prepared from previously immobilized muscle greatly increased phosphorylase activity (Table V). With the patient's muscle, on the other hand, these compounds did not increase enzymatic activity. Furthermore, the patient's muscle did not appear to contain phosphorylase inhibitors or increased activity of the phosphorylase-rupturing enzyme (50, 51), since the muscle homogenate from the patient did not reduce the enzymatic activity of normal muscle preparations (Table V).

In contrast to this defect in the phosphorylase system, lactate formation from phosphorylated hexoses was not impaired *in vitro* (Table VI). Likewise, *in vivo*, oral and intravenous administration of glucose and intravenous injection of insulin (Table II) resulted in a significant increase in venous lactate concentration; this was associated with a concomitant reduction in serum phosphorus and NEFA. Moreover, the hyperglycemia following injection of glucagon was accompanied by similar changes in lactate, phosphorus and NEFA levels (Table I). These observations indicated active utilization of glucose in the periphery. It should be noted that the fall in serum phosphorus, following injection of glucose and insulin (Table II) and of glucagon (Table I), exceeded that which is commonly observed in normal individuals under similar conditions; whether this reflected participation of an ancillary mechanism of phosphorus metabolism could not be assessed on the basis of the available data.

Additional evidence for active utilization of blood glucose by the patient's muscles was provided by the observation that, on the treadmill, infusion of glucose and insulin prevented the occurrence of cramps and greatly increased the patient's work performance (Figure 2). It could not be determined, however, whether under these circumstances, peripheral uptake of glucose in the patient was comparable to that which would be expected in normal individuals subjected to similar treatment. Similarly, no information was obtained to indicate whether, in the fasting patient, the assimilative capacity of muscle for blood glucose differed from that of normal muscles. The rapid onset of cramps in the patient's muscles on moderate exertion may suggest that in addition to impaired glycogenolysis, uptake and/or utilization of blood nutrients may have been reduced. One may consider the possibility that, in contracting muscle, glycogenolysis may exert a priming effect in initiating increased assimilation of glucose and other nutrients from the blood. This could be achieved in various ways, perhaps by increasing blood flow through the muscle, by facilitating transport of nutrients across the cell membrane, or by modifying their metabolic disposition in the muscle cell. A possible approach to long term management of this patient may be maintenance of a hyperglycemic state in the presence of adequate insulin.

The defect in the phosphorylase system apparently did not involve the liver, as administration of epinephrine and glucagon evoked a significant degree of hyperglycemia, indicating adequate breakdown of glycogen and output of glucose by the liver (26). Moreover, the liver was not enlarged and hepatic function, including glucose tolerance, appeared to be within normal limits. The glycogen content of circulating leukocytes was not increased and there was no demonstrable abnormality of glycolysis in peripheral blood (47).

In agreement with the chemical analysis, the histological findings in the muscle demonstrated the presence of increased concentrations of glycogen. The latter was predominantly located directly under the sarcolemma, but to a lesser degree was present diffusely throughout the muscle fibers. The presence of a high glycogen content in the patient's muscle suggested that only the breakdown but not the formation of glycogen may be impaired. This is consistent with recent reports by Leloir and Cardini (52), Villar-Palasi and Larner (53) and Robbins, Traut and Lipmann (54), that phosphorylase is involved only in glycogenolysis, while synthesis of glycogen occurs via the uridine nucleotide pathway. The formation of glycogen in the patient's muscle is presently under investigation; the results of this study are reported in a separate communication (55).

The disturbance described in this patient appears to be a very rare disorder of muscle metabolism. In 1951, McArdle (56) reported the find-

ings in a 30 year old patient, who exhibited a similar type of muscle dysfunction, although excretion of myoglobin was not observed. Exercise and ischemic exercise failed to produce increased lactate concentrations, whereas epinephrine resulted in both hyperglycemia and elevation of lactate levels. From this, it was concluded that the patient "suffered from a disorder of carbohydrate metabolism, affecting chiefly if not entirely, the skeletal muscles and preventing the breakdown of glycogen to lactic acid." Biochemical or histological investigations of muscle tissue were not performed.

In a recent abstract, Pearson, Rimer and Mommaerts reported studies on another case with similar features (57). This patient was 19 years old and "had a life-long history of progressive weakness upon usage of exercised muscles and severe cramps if exertion was intense or prolonged." Muscle wasting was not present and myoglobinuria was not observed. Venous lactate concentration did not rise following exercise, and biochemical studies of muscle tissue showed virtual absence of phosphorylase activity.

In addition to these reported cases, one of us (R. M.) has observed five other patients with similar clinical and laboratory findings, but biochemical studies of muscle tissue have not yet been performed. Two of the patients were sisters, but this is the only evidence to suggest a hereditary nature of the disorder. The patient presently reported has nine living siblings and one sister, who died from tuberculosis, none of whom had any evidence of disturbed muscle function.

The history of the patient suggests that his clinical course may be somewhat arbitrarily divided into three distinct periods. The first included childhood and adolescence when the patient's only complaint was increased fatigability. The period from age 20 to approximately 40 was characterized by severe cramps on exertion associated with transient myoglobinuria, but persistent weakness and wasting of muscles was not prominent. During the third period, beginning at approximately 40 years of age, cramps and myoglobinuria became gradually less conspicuous, but weakness and wasting of individual muscle groups began to appear with increasing severity. Associated with this loss of muscle mass was a marked elevation of creatine excretion in the urine (Table III). It is apparent that the features of the two patients reported in the literature resembled those of our patient during the second clinical period. McArdle's case (56) was 30 years of age and had severe cramps on exertion, but persistent weakness, wasting of muscles and creatinuria were absent. Pearson's patient (57) was 19, and although intense or prolonged exercise produced cramps, exercise tolerance in the fasting state was much higher than in our patient. In neither of the two reported cases was myoglobinuria observed, but unless specifically sought, this may have passed unnoticed.

Several features of the present patient remain unexplained. It is not clear why the pattern of muscle cramps on exertion did not develop until the beginning of the third decade; this may perhaps suggest that the energy metabolism of growing muscle may quantitatively differ from that of adult muscle. Moreover, it is not understood why wasting of muscles did not occur until later in life and why some muscle groups were more affected than others, while the calf muscles showed actual hypertrophy. Finally, it was of interest that no evidence was found to suggest impairment of myocardial function.

SUMMARY AND CONCLUSIONS

1. Clinical manifestations and laboratory investigations of a 54 year old male patient, who had suffered for the past 35 years from chronic, progressive myopathy, are reported. The outstanding feature of this patient was his inability to perform moderate degrees of muscular exercise, even over short periods of time, whereas minimal muscular work was tolerated almost without limitation. Moderate exercise of a few seconds' duration resulted in prolonged, painful cramps of the involved muscle groups, associated with tissue necrosis and transient myoglobinuria.

2. Exercise and ischemic exercise, sufficient in extent to produce a cramp, failed to result in the expected rise in venous lactate concentration. This finding suggested a defect involving the glycolytic mechanisms in the muscle.

3. Glycogen content of the muscle was found to be approximately five times increased; in spite of this, incubation of muscle homogenate produced four times less lactate than in control preparations.

This defect could be corrected by addition of crystalline phosphorylase.

4. With glucose-1-phosphate as substrate, lactate formation in the patient's muscle was comparable to that of control preparations. These observations suggested that the disturbance involved the phosphorylase system, which catalyzes the breakdown of glycogen to glucose-1-phosphate.

5. In the patient's muscle, phosphorylase activity was found to be absent, even in the presence of adenylic acid or after preincubation of tissue with epinephrine.

6. Treatment of the patient with epinephrine or glucagon resulted in hyperglycemia and increased serum lactate levels, associated with marked reduction in serum phosphorus.

7. Infusion of glucose and insulin produced increased venous lactate concentration and reduced serum levels of phosphorus and nonesterified fatty acids. Associated with this was a marked increase in exercise tolerance.

8. These findings indicate a defect in the phosphorylase system of the muscle, which precludes breakdown of glycogen and eliminates anaerobic glycogenolysis as a source of energy for muscular contraction. Muscular work is limited by the energy which can be derived from nutrients diffusing from the blood into the muscle cell. Contraction beyond these limits results in cramps, muscle necrosis and myoglobinuria.

9. A disturbance of similar nature has been described in two other patients; in addition, five patients with myopathy and similar clinical findings were observed, but details have not yet been reported. The possible hereditary nature of this rare syndrome remains to be investigated.

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ADDENDUM

For the last four months, the patient has been treated with intramuscular injections of 1 mg. glucagon three times daily. Glucagon is given simultaneously with or shortly following meals and results in sustained hyperglycemia, lasting from 90 to 120 minutes. During this time, the patient notices a significant improvement in his ability to perform muscular work. For example, one hour after injection of glucagon, he was able to walk on the treadmill for 17 minutes (angle 2°, speed two miles per hour), while after injection of saline, cramps occurred in both legs in less than two minutes, necessitating termination of the exercise.

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THE REACTION OF THE LUPUS ERYTHEMATOSUS (L.E.) CELL FACTOR WITH DEOXYRIBONUCLEOPROTEIN OF THE CELL NUCLEUS

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Previous reports have presented evidence that the lupus erythematosus (L.E.) cell factor reacts with deoxyribonucleoprotein of the cell nucleus, and that deoxyribonucleic acid (DNA) is essential for the reaction (1, 2). More recently, a number of other serum factors have been identified in systemic lupus erythematosus (SLE) which react with other constituents of the nucleus. These include isolated DNA, purified histone and a material extractable from the nucleus with isotonic buffers which is neither nucleic acid nor histone (3, 4).

These reactions with nuclear constituents have been demonstrated by standard immunological techniques such as complement fixation (5, 6), precipitation (7, 8), hemagglutination (9), latex agglutination (10) and passive cutaneous anaphylaxis (11). The serum factors, including the L.E. cell factor, migrate with γ-globulin on electrophoresis and sediment with γ-globulin on centrifugation. These results suggest that the L.E. cell factor is but one of a group of antibodies to different components of the nucleus which arise in SLE. However, knowledge of the precise reactivities of the antinuclear factors is necessary in order to establish that they are antibodies and to permit study of their origin and biologic roles. The L.E. cell factor provides an example of the need for such information. Already three different nuclear constituents have been suggested as the substance with which this factor reacts: intact deoxyribonucleoprotein (1, 2, 12), DNA alone (13) and a protein of the nucleus but not DNA (14).

If the L.E. cell factor is an antibody, it should exhibit a particular antigenic specificity and, when isolated, should be indistinguishable from typical human antibody γ-globulin. The work reported in this paper comprises an effort to identify the nuclear component upon which the L.E. cell factor acts, and to isolate the factor and define certain of its properties. The term L.E. cell factor is used to signify only that factor which induces formation of L.E. cells, and none of the other antinuclear factors.

Because fractionation of the cell nucleus is still in a primitive stage and because many of the methods employed in this study have not been extensively used and verified, the experimental details will be given together with the results.

MATERIALS AND GENERAL METHODS

Sera were collected and stored under sterile conditions at 4° C., usually with 1:10,000 concentrations of merthiolate.

Nuclei were obtained from calf thymus glands at 4° C. by the method of Mirsky and Pollister (15). Glands were minced with scissors, homogenized in a solution of 0.25 M sucrose and 0.003 M $CaCl_2$ in a Waring blendor at a speed which did not disrupt the nuclei, filtered through gauze and flannel and the nuclei separated by centrifugation. The resulting nuclei were intact microscopically and had minimal cytoplasmic contamination. For storage, nuclei were lyophilized.

Nucleoprotein was prepared from unlyophilized nuclei by extraction overnight at 4° C. with 1 N NaCl (16). The resulting viscous suspension was centrifuged at 78,000 × G for 90 minutes in the Spinco "L" ultracentrifuge and the clear viscous supernate solution of the nucleoprotein stored at 4° C. Such solutions remained stable for at least 30 days under these conditions. Nucleoprotein was obtained for experimental use by diluting the stock solution with six volumes of cold distilled water, thus bringing it to physiologic salt concentration. Silvery nucleoprotein strands precipitated. These strands were separated by centrifugation and homogenized in a Teflon tissue grinder in isotonic saline. Equal volumes of the homogenate were then removed, spun, and pellets of solid, particulate nucleoprotein used for absorption. In general, nucleoprotein pellets containing 1 to 1.5 mg. DNA were used to absorb 0.5 ml. of serum, although pellets half that size are adequate to absorb completely 0.5 ml. of active serum if an incubation time of 45 minutes at 37° C. were allowed.

Human white cell nuclei were prepared from leukocytes from patients with granulocytic and monocytic leukemia. The above methods were used except that the cells were disrupted in a Teflon tissue grinder in a solution of 0.25

TABLE I

*Typical examples of absorption of protein from systemic lupus erythematosus (SLE) serum by nucleoprotein**

		Material absorbed					
		Saline	Normal serum	Hyper-γ-glob. serum	SLE serum	SLE serum	SLE serum
L.E. prep. with absorbed serum					0	0	±
Analysis of pellet after absorption	Total DNA† *mg.*	1.00	0.81	0.81	0.92	1.00	0.94
	Total protein *mg.*	0.90	0.70	0.70	1.23	1.56	1.17
	Protein/DNA† ratio	0.90	0.86	0.86	1.33	1.56	1.24

* Equal aliquots of serum or saline were absorbed with pellets of nucleoprotein for 45 minutes at 37° C. Pellets were then washed and total DNA and protein determined. Data are expressed in terms of pellets containing 1 mg. DNA at start of experiment.

† DNA = deoxyribonucleic acid.

M sucrose, 0.003 M $CaCl_2$ and 0.2 per cent citric acid. Human leukocyte nucleoprotein was obtained from unlyophilized nuclei in the manner employed for calf thymus nucleoprotein.

Calf thymus DNA was purchased from the Mann Research Laboratories, Inc., New York, and showed the following analyses: C, 37.18 per cent; N, 16.54 per cent; P, 9.91 per cent. DNA concentrations were determined by absorption at 260 mμ in a Beckman Spectrophotometer. The material to be analyzed was heated to 70° C. for 30 minutes in 10 per cent perchloric acid, centrifuged and the clear supernate used for assay.

Protein concentrations were determined by the modified method of Folin-Ciocalteau (17). Solid materials were dissolved in 0.1 N NaOH prior to removal of aliquots for protein determination.

Calf thymus histone prepared by acid extraction of nucleoprotein was purchased from Worthington Chemical Corporation, Freehold, New Jersey. Lysine-rich histone prepared from calf thymocytes was kindly donated by Dr. Alfred Mirsky of the Rockefeller Institute. Standard curves for determining histone concentration were constructed using solutions of these histones and the Folin-Ciocalteau method.

Once crystallized pancreatic deoxyribonuclease (DNase) was obtained from Worthington Chemical Corporation. All experiments employing this enzyme were done with fresh solutions of enzyme in phosphate buffer, ionic strength 0.1, pH 6.8, and containing 1 μMole of magnesium for every 0.1 mg. of DNase.

L.E. cell preparations were made by the method of Snapper and Nathan (18) using a mixture of the material to be tested and fresh normal human blood. This method permits localization of the L.E. cells in a small area which facilitates reading and comparing of slides. Only sera which formed large numbers of L.E. cells were studied. Removal or inhibition of the L.E. cell factor in these sera resulted in a striking reduction or complete disappearance of L.E. cell formation which could be readily identified. Thus the results could be reported in most instances as highly positive (++++), negative (0), or very weakly positive (±). No satisfactory method for quantification of L.E. cell formation exists.

Complement fixation was studied by the method of Casals and Palacios (19) as applied to this system (5). Two 100 per cent units of complement were used and visual endpoints recorded with appropriate serum and antigen controls. When materials containing DNase were used for complement fixation, sodium citrate was added in excess of the magnesium concentration and all reagents employed were free of both calcium and magnesium.

Precipitin reactions in agar plates were conducted by the method of Ouchterlony (20) employing 0.5 per cent agar in isotonic NaCl or appropriate buffer. Double diffusion precipitin reactions in agar were conducted as outlined previously (8).

Fluorescent antibody studies were conducted with Drs. Mellors and Ortega utilizing techniques previously described (21).

Rabbit antisera to Red Cross or Lederle Fraction II human γ-globulin, Red Cross human albumin and whole normal human serum were employed for immunologic assays. Antiserum to 19S human γ-globulin was prepared by absorbing rabbit antiserum to normal human γ-globulin (separated by electrophoresis) with Fraction II γ-globulin, or by immunizing rabbits with isolated human macroglobulins. Estimations of γ-globulin were made by the capillary tube precipitation method, with gradation of the interface precipitate from trace to (++++) amounts. Quantitative determinations of γ-globulin were made by the standard precipitin method, using 0.5 ml. of the appropriate dilutions of γ-globulin solution, 0.5 ml. buffer and 0.05 ml. of the standardized antiserum.

EXPERIMENTS AND RESULTS

Adsorption of γ-globulin and L.E. cell factor by nucleoprotein

Examples of the highly specific absorption of protein from SLE serum by nucleoprotein are

seen in Table I. Equal amounts of homogenized calf thymus nucleoprotein were used to absorb equal volumes of isotonic saline, normal human sera, sera from patients with hypergammaglobulinemia due to liver disease, and SLE sera. After absorption, the nucleoprotein was washed until the wash fluid was free of protein and the pellets were then analyzed for total protein and total DNA. The ratio of protein to DNA remained about the same in that nucleoprotein which was used to absorb saline, normal serum, and serum with hypergammaglobulinemia. However, this ratio was increased greatly in the nucleoprotein pellets used to absorb SLE serum. Ratios greater than 0.91, which was that of unaltered nucleoprotein, were found only after exposure to SLE serum. The total amounts of protein and DNA were reduced in the pellets used to absorb normal sera and hypergammaglobulinemic sera, though the ratio remained the same. This was due to dissolving of nucleoprotein during absorption. It is possible that a protective effect of the adherent γ-globulin prevented a similar degree of solution in the SLE serum.

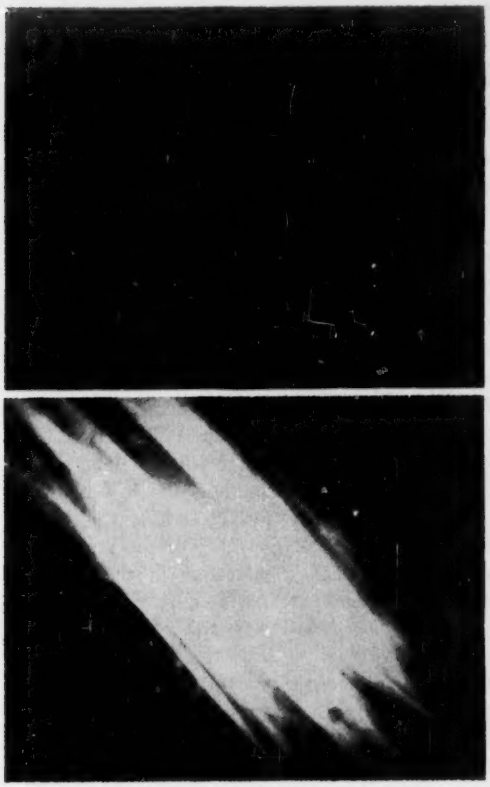

FIG. 1. ABSORPTION OF γ-GLOBULIN BY NUCLEOPROTEIN EXPOSED TO SLE SERUM

Identification of γ-globulin on nucleoprotein by fluorescent antibody method. Upper frame is photograph of nucleoprotein strands exposed to normal serum, washed and stained with fluorescein-labeled rabbit antibody to normal human γ-globulin. Lower frame is photograph of nucleoprotein strands exposed to SLE serum and treated similarly.

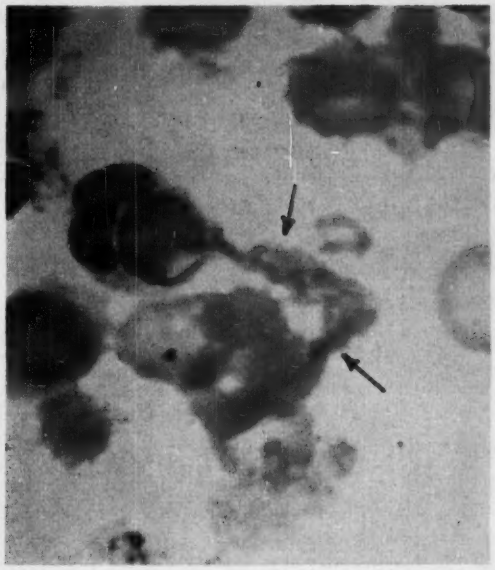

FIG. 2. PHAGOCYTOSIS OF A NUCLEOPROTEIN STRAND COATED WITH L.E. CELL FACTOR

Strands of nucleoprotein were used to absorb SLE serum, washed and incubated at 37° C. for one hour with fresh, normal human leukocytes in normal plasma. Smears were then made. Arrows indicate nucleoprotein strand being ingested by the leukocyte at the left. Many erythrocytes are present. After staining with Wright's stain, the nucleoprotein was light blue and the cell nuclei were dark blue.

The absorption of γ-globulin from SLE serum by nucleoprotein serum may be seen in Figure 1. In this experiment, strands of nucleoprotein were used to absorb both normal and SLE serum. The nucleoprotein strands were then washed six times and stained with antibody to normal human γ-globulin labeled with fluorescein. In the upper black frame nucleoprotein exposed to normal serum was stained, and none of the fluorescent

TABLE II

*Recovery of lupus erythematosus (L.E.) cell factor from complex with nucleoprotein**

1	2 56° Eluate from complex	3 Supernate from DNase digestion of complex	4 56° Eluate from digestion residue	5 Residue from 56° elution in Column 4	6 2N NaCl 37° C. eluate from digestion residue	7 Residue from 2N NaCl elution in Column 6
% Histone	±2	15–20	5–10	75–85		
% DNA	±2	75–85	5–10	10–20		
γ-Globulin	±	++	+++	present	+++	present
L.E. cell factor	±	±	++++		++++	
Complement fixation	0	DNA, nuclei, nucleoprotein	nuclei, nucleoprotein			
Precipitation with DNA	0	0	0			

* Complexes were made by incubating pellets of nucleoprotein containing 1.3 mg. of deoxyribonucleic acid (DNA) with 0.5 ml. of serum. Liberated and residual DNA and histone were determined on identical aliquots of nucleoprotein run in parallel but incubated only with saline. Per cent DNA and per cent histone refer to per cent of total in original pellet. For further details, see text.

antiglobulin was bound. The nucleoprotein in the lower frame was passed through SLE serum and is seen to be brilliantly fluorescent, indicating that it had absorbed γ-globulin from the serum which then reacted with the fluorescent antibody.

After absorption with nucleoprotein, SLE serum was no longer able to induce L.E. cell formation, indicating specific removal of the L.E. cell factor. The nucleoprotein from this absorption became susceptible to phagocytosis by viable white cells with the formation of inclusion bodies very similar to the inclusion bodies of typical L.E. cells (Figure 2). The inclusion bodies appeared homogeneous and stained light purple with Wright's stain. No such phagocytosis occurred with nucleoprotein exposed to normal serum.

Recovery of L.E. cell factor from the complex with nucleoprotein

Table II summarizes these experiments. Column 1 lists the assays made on solutions obtained during efforts to recover the factor from nucleoprotein after absorption of SLE serum; the other columns give the results of these assays after the steps indicated at the top of the columns. Column 2 shows that elution at 56° C. in isotonic saline did not liberate significant amounts of the constituents of nucleoprotein, of γ-globulin, or of L.E. cell factor. Column 3 indicates that after digestion of the nucleoprotein complex with deoxyribonuclease, a small amount of the histone, a large amount of the original DNA and considerable γ-globulin were recovered. This γ-globulin had little if any capacity to induce formation of L.E. cells but was capable of fixing complement with isolated DNA, isolated nuclei and nucleoprotein.

If the residue remaining after DNase digestion was eluted at 56° C. in isotonic saline, an additional large amount of γ-globulin was recovered which possessed great ability to induce L.E. cell formation. This γ-globulin was capable of fixing complement with nuclei and nucleoprotein but not with isolated DNA (Column 4). The residue which remained after both DNase digestion and elution at 56° C. contained most of the original histone, a small amount of the original DNA and a certain amount of γ-globulin (Column 5). An alternative procedure to the elution at 56° C. in isotonic saline was elution at 37° C. in 2 N saline. This procedure also yielded considerable γ-globulin and much L.E. cell factor from the residue remaining after DNase digestion of the complex. Columns 6 and 7 summarize this type of elution.

Identical results were obtained with each of 20 SLE sera studied by this method, despite the fact that these sera contained widely differing groups of other antinuclear antibodies. Thus the recovery of L.E. cell factor from nucleoprotein required first digestion of the DNA by DNase and then elution of the factor from the residual material, which is primarily histone and nonhistone protein. This sequence of procedures was therefore used as a standard method throughout subsequent experiments designed to study the reaction of the L.E. cell factor with nucleoprotein.

A number of the techniques used in the experiments in Table II require further explanation. *1)*

The attachment of L.E. cell factor to the residue after DNase digestion is weakened by heat. Not only is the factor liberated at 56° C., but small amounts of it are liberated at room temperature and below. Therefore the digestions with DNase were conducted at 4° C. for 15 to 18 hours to minimize elution of L.E. cell factor from the digestion residue during the course of the digestion. Judged by removal of DNA, digestion at 4° C. for 15 hours is as effective as digestion at 37° C. for one hour. The DNA remaining after this digestion appears to be an "indigestible" residue; further exposure to DNase does not lead to further removal of DNA. *2)* Assay of the DNase supernate for L.E. cell formation proved difficult initially. The DNase present in the supernate destroyed the nuclei of the dead "substrate" leukocytes during L.E. cell tests and thus prevented formation of L.E. cells. However, the enzyme was inactivated by heating to 65° C. for 45 minutes while the L.E. cell factor was not. Therefore, DNase supernates were heated to 65° C. for 45 minutes prior to assay of their ability to form L.E. cells. Such heated supernates were shown to be both incapable of inhibiting L.E. cell formation when mixed with solutions of L.E. cell factor and incapable by themselves of causing more than occasional, minimal formation of L.E. cells. This latter activity appeared to be little more than would dissociate if the residue from enzyme digestion were allowed to stand overnight at 4° C. in the absence of enzyme. *3)* The γ-globulin still adherent to the residue of the complex after both DNase treatment and 56° C. elution was identified by a modified γ-globulin consumption method. The insoluble residue was divided into equal aliquots; one was incubated with normal rabbit serum and the other with rabbit antiserum to human γ-globulin for one hour at 22° C. and overnight at 4° C. The tubes were then spun and the pellets washed twice. Total protein was determined on each pellet; much more was present in the pellet incubated with antiserum. This indicates adsorption of antiglobulin protein and therefore also the presence of γ-globulin on the residue. This γ-globulin could not be removed from the residue by further digestion with DNase, further elution at 56° C. or elution in 2 N NaCl. It therefore has not been characterized.

Evidence obtained with the two step recovery technique underscored the specificity of the adsorption of γ-globulin from SLE serum by nucleoprotein. If normal serum was absorbed with nucleoprotein, which was then digested with DNase and the residue eluted at 56° C. with isotonic saline, no γ-globulin was recovered. If the same was done with sera from other diseases containing levels of γ-globulin much higher than those present in SLE sera, only a trace or no γ-globulin was recovered.

Identification of constituents of nucleoprotein which react with the L.E. cell factor

In these and subsequent experiments, five sera were used which contained large amounts of L.E. cell factor and varying types of other antinuclear factors. The results obtained were the same for the five sera.

Prior to absorption of SLE serum, nucleoprotein was digested with DNase to remove the DNA, or extracted with 0.1 N HCl to remove histone. Both procedures left insoluble residues which were then employed to absorb the serum. The amounts of nucleoprotein, present at the starting point, and of serum used for absorption were kept

TABLE III

*Effect of removal of deoxyribonucleic acid (DNA) or histone on ability of nucleoprotein to absorb L.E. cell factor**

	Untreated control nucleoprotein	Nucleoprotein with DNA removed	Nucleoprotein with histone removed
DNA before absorption (*mg.* total in pellet)	1.32	0.19	1.32
Protein before absorption (*mg.* total in pellet)	1.10	0.83	0.20
L.E. cell formation absorbed serum	0	++++	++++
DNA after absorption (*mg.* total in pellet)	1.32		1.28
Protein after absorption (*mg.* total in pellet)	1.70	0.64	
L.E. cell formation DNase supernate	±	0	0
L.E. cell formation 56° supernate	++++	+	+

* The nucleoprotein pellet was digested at 37° C. for one hour in 0.5 ml. of a solution of 0.2 mg. DNase per ml. of buffer, or was extracted at room temperature in 1 ml. of 0.1 N HCl for 15 minutes. The residues were washed three times before use for absorption.

constant. Results are seen in Table III. In the column labeled "Untreated control nucleoprotein," the results of a typical absorption with untreated nucleoprotein are given. The serum was unable to induce L.E. cell formation after absorption and highly active L.E. cell factor could be recovered from the nucleoprotein. However, nucleoprotein from which DNA or histone was largely removed was able to absorb only very small amounts of L.E. cell factor. In the experiment in which histone was removed, the amount of DNA present after the attempted absorption was the same as that present initially, demonstrating that DNA had not dissolved during the absorption. The histone residue after removal of DNA dissolved partially on absorption. However, this experiment was done repeatedly using larger amounts of histone residue with the same volume of serum, and only very slight absorption of L.E. cell factor occurred.

By the methods employed, it was never possible to remove from the nucleoprotein all of the DNA by digestion or all of the protein by extraction. The absorption of small amounts of L.E. cell factor by these altered nucleoproteins was presumably due to a small residual amount of nucleoprotein which resisted enzyme digestion or acid extraction.

Because nucleoprotein from which either DNA or histone had been removed had a greatly reduced capacity to absorb L.E. cell factor, an effort was made to recombine the separated portions and to determine whether or not a "reconstituted" nucleoprotein regained its reactive capacity. After removal of DNA with DNase, efforts at reconstitution by incubation of the washed residue with a solution of purified DNA equal in amount to that removed were unsuccessful. Little DNA combined with the residue and the product did not differ significantly from the residue before recombination. Reconstitution was achieved, however, after extraction of histone from the nucleoprotein by acid. In these experiments nucleoprotein pellets containing 1.3 mg. of DNA were extracted for 15 minutes at room temperature with 1 ml. of 0.1 N HCl. This removed about 90 per cent of the protein. The extract was then dialyzed overnight against isotonic NaCl to remove acid and the next day the extracted protein was recombined with the residue in a homogenizer at room temperature. Residue and extracted protein recombined quantitatively yielding a product almost identical in composition to the original nucleoprotein. This reconstituted nucleoprotein gave evidence of some return of reactive capacity. It was capable of absorbing some L.E. cell factor from serum, and γ-globulin and L.E. cell factor could be recovered from the reconstituted nucleoprotein after absorption. However, under the conditions we have employed we have not succeeded in obtaining return of more than about 50 per cent of the original reactivity; when compared with normal nucleoprotein on a weight basis, twice as much reconstituted nucleoprotein was necessary to absorb completely a given amount of serum.

Next, an effort was made to determine whether other long chain acids or basic proteins could be substituted for the DNA or histone of the nucleoprotein. These results with "synthetic" complexes are summarized in Table IV. The "synthetic" nucleoprotein which is obtained from a mixture of purified DNA and isolated histone possesses some capacity to absorb L.E. cell factor, but not the capacity of an equivalent amount of control nucleoprotein. Pellets of this "synthetic" nucleoprotein contained slightly more DNA than the control nucleoprotein. However, it proved extremely difficult to duplicate in the "synthetic" DNA-histone the exact protein to DNA ratio found in extracted nucleoprotein. The latter ratio was consistently 0.91 by our methods of assay. Despite repeated experiments with wide variation in the amounts of histone and DNA mixed, the resulting "synthetic" nucleoprotein possessed a ratio close to 1.0, indicating approximately 10 per cent more histone per unit of DNA than in extracted nucleoprotein. Substitution of protamine for histone in the reaction with purified DNA gave a product with the appearance of extracted nucleoprotein but completely devoid of reactivity. In this case weight-combining ratios of protamine to DNA near 0.5 were used because the molecular weight of protamine is estimated to be about one-third that of histone. It was assumed that with such a ratio, the saturation of binding sites on the DNA with protamine would more closely approximate the saturation of DNA with histone in control nucleoprotein. Here too, we could not achieve a ratio lower than 0.5. Substitution of heparin, hyaluronic

TABLE IV

*Absorption of L.E. cell factor by "synthetic" nucleoprotein, long chain acid complexes with histone and protamine, and DNA alone**

		Control nucleo-protein	Synthetic DNA-histone	Synthetic DNA-protamine	Heparin-histone	Heparin-protamine	Hyaluronic acid-histone	Poly-ethylene sulfonate-histone	DNA alone
L.E. prep. with abs. serum		0	++	++++	++++	++++	++++	++++	++++
Supernate DNase digestion	γ-Globulin	++	++	+			ft. tr.	0	++
	L.E. prep.	±	+	0					±
Supernate 56° elution	γ-Globulin	++	+	tr.	tr.	0	0	0	0
	L.E. prep.	++++	++	0		0	0		0

* The synthetic substances were made by mixing saline solutions of the components at room temperature, whereupon precipitation occurred. The control nucleoprotein contained 1.2 mg. DNA with a protein:DNA ratio of 0.91. The DNA-histone contained 1.4 to 1.6 mg. DNA with a protein:DNA ratio of near 1.0. The DNA-protamine contained up to 2 mg. DNA with a protein:DNA ratio near 0.5. The heparin-histone, heparin-protamine, hyaluronic acid-histone and polyethylene sulfonate-histone precipitates were obtained by mixing solutions containing 2 mg. of each reactant. The precipitates contained 1 to 1.5 mg. of the protein. All precipitates were used to absorb 0.5 ml. aliquots of the same L.E. serum. 1.2 mg. pure DNA was used for absorption.

acid or polyethylene sulfonate for DNA in the reaction with histone also yielded solid complexes which could be used for absorption but which were inactive. Thus it appears that not only are both the DNA and the histone of the nucleoprotein necessary for the reaction with L.E. cell factor but also that the specific combining configuration present in the nucleoprotein extracted from nuclei is most efficient, and was apparently not obtained in the various reconstituted or "synthetic" nucleoprotein preparations.

For purposes of comparison, data resulting from attempts to absorb L.E. cell factor with highly purified DNA are given in the last column of Table IV. This experiment was done in two ways. First, DNA in amounts equal to that normally present in the standard pellet of homogenized nucleoprotein was added to SLE serum and allowed to incubate for 30 minutes at 37° C. Minimal agitation was used so that the DNA did not dissolve, but simply formed a gelatinous mass. This was removed by centrifugation after absorption, washed twice at 4° C. and treated with DNase in the usual manner. The second method was to add to SLE serum the same amount of DNA, but then conduct the absorption at 37° C. with considerable agitation so that the DNA dissolved in the serum. After absorption, an amount of protamine was added sufficient to precipitate most of the DNA. This precipitate formed instantaneously and was promptly spun out of the serum. The serum was then analyzed for L.E. cell activity and the DNA-protamine precipitate digested in the usual way. In neither instance was the DNA capable of absorbing any significant amount of L.E. cell factor, though in each case it absorbed γ-globulin which was liberated after the nuclease digestion alone.

Additional evidence supporting the participation of histone in the reaction with the L.E. cell factor emerges from the failure of the L.E. cell factor to displace histone upon combination with nucleoprotein. A solution of purified L.E. cell factor, recovered from nucleoprotein, was absorbed with a fresh aliquot of nucleoprotein which was then removed by centrifugation. After absorption, all but a trace of the γ-globulin and 90 per cent of the total protein had been removed,

TABLE V

Absence of histone liberation from nucleoprotein during combination of L.E. cell factor and nucleoprotein

	γ-Globulin	Total protein	L.E. cell formation
		mg./0.5 ml.	
L.E. cell factor solution	+++	0.310	++++
L.E. cell factor solution after absorption	tr.	0.032	±
Supernate from DNase digestion of absorption pellet	tr.	0.050 (minus DNase)	±
Supernate from 56° elution digestion residue	++	0.110	+++

TABLE VI

*Inhibition of reaction of L.E. cell factor with nucleoprotein**

		Untreated control nucleoprotein	Nucleoprotein pretreated with histone	Nucleoprotein pretreated with protamine	Nucleoprotein pretreated with atabrine
L.E. prep. with absorbed serum		0	++++	++++	+++
Supernate DNase digestion	γ-Globulin	++	0	0	+
	L.E. prep.	±			
Supernate 56° elution	γ-Globulin	++	0	0	tr.
	L.E. prep.	++++	0	0	+

* Nucleoprotein pellets were incubated for 60 minutes at 37° C. in solutions of histone, protamine or atabrine, washed and used to absorb SLE serum. See text for details.

leaving behind a small amount of L.E. cell factor (Table V). The fact that the total protein of that solution was reduced by at least 90 per cent suggests that the L.E. cell factor does not displace histone from nucleoprotein. After this second absorption, L.E. cell factor could again be recovered from the nucleoprotein in the usual way. The values for total protein given in Table V include both γ-globulin and undetermined amounts of histone. The failure to recover all of the L.E. cell factor absorbed by the nucleoprotein may be explained by the inability of the procedures to liberate all γ-globulin from the nucleoprotein complexes (Table II).

Inhibition of the reaction between L.E. cell factor and nucleoprotein

It had previously been found that exposure of cell nuclei to protamine or atabrine, which react with DNA and nucleoprotein to form stable compounds (22), prevented subsequent reaction of these nuclei with the L.E. cell factor (1). Similar experiments were conducted with nucleoprotein using histone, protamine and atabrine. Nucleoprotein pellets containing 1.2 mg. DNA were incubated for one hour at 37° C. in 1 ml. of isotonic saline solutions containing 1 mg. of histone or protamine or in a solution of isotonic saline saturated with atabrine. The pellets were then washed six times and used to absorb SLE serum. Little or no absorption of L.E. cell factor occurred (Table VI). The nature of the reaction of the inhibiting substance with nucleoprotein was not the same in each case. In histone solution, the nucleoprotein absorbed histone until there was an increase of approximately 70 per cent in the total histone of the nucleoprotein. No such absorption occurred in protamine solution; the total protein of the nucleoprotein diminished slightly. However, after incubation with nucleoprotein, the protamine solution contained a nondialyzable protein not present prior to the incubation. Because protamine is dialyzable and histone is not, it appears that protamine displaced some histone from the DNA, thus changing the original DNA-histone nucleoprotein at least partly into a DNA-protamine nucleoprotein. Atabrine stained the nucleoprotein yellow. For technical reasons it was not possible to establish clearly whether atabrine displaced histone. However, atabrine is known to react both with DNA and with albumin (22), and it is possible that it reacted with either DNA or histone of the nucleoprotein or both.

Failure of L.E. cell factor of one patient to react with nucleoprotein saturated with the factor of another patient

Successive aliquots of serum of one patient were absorbed with the same nucleoprotein pellet until no additional L.E. cell factor could be removed from the serum. The pellet was then washed and used to absorb another serum. In no instance was the pellet, saturated with the L.E. cell factor of one patient, able to absorb L.E. cell factor from the serum of another patient. These experiments were done with various combinations of the five sera used in the other experiments.

Immunological characteristics and sedimentation behavior of the L.E. cell factor

The data presented in this section were derived from the studies done with whole SLE serum and with L.E. cell factor recovered from nucleoprotein. Normal human γ-globulin consists of two main molecular species; approximately 90 to 95 per cent has a molecular weight of 160,000 and a sedimentation rate of approximately 7S, while the remaining 5 to 10 per cent has a molecular weight of about 1,000,000 and a sedimentation rate of approximately 19S (23). Figure 3 and Table VII indicate that the L.E. cell factor possesses the immunologic characteristics of typical human 7S γ-globulin. In Figure 3A, the isolated L.E. cell factor from one patient in Well 1 forms a single merging band of precipitate against antiserum to whole normal human serum and antiserum to normal human 7S γ-globulin. In Figure 3B the L.E. cell factor from another patient in Well 4 forms similar single bands of identity with normal 7S γ-globulin as both diffuse against the same antisera. The L.E. cell factors isolated from 20 different sera have all reacted with antiserum to normal human 7S γ-globulin. At no time has any serum protein other than γ-globulin been found in the L.E. cell factor solutions. Occasionally a small amount of the 19S γ-globulin has appeared, the significance of which is obscure.

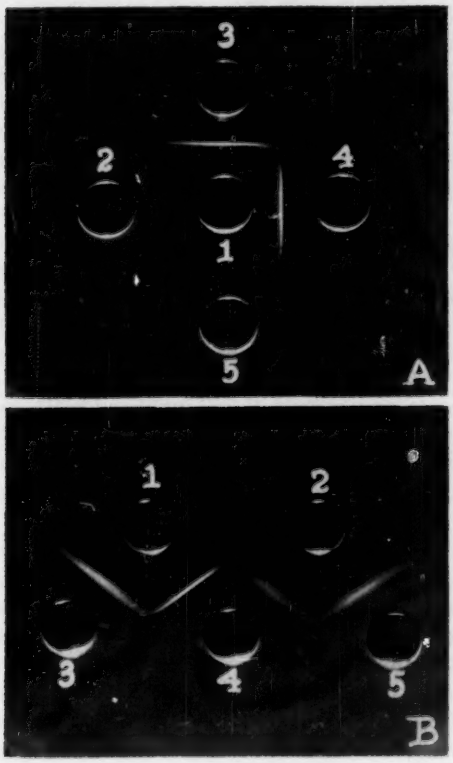

FIG. 3. REACTION OF PURIFIED L.E. CELL FACTOR WITH VARIOUS ANTISERA

3A. L.E. cell factor from Patient Ter. in Well 1; Well 2, antiserum to normal human 19S γ-globulin; Well 3, antiserum to normal whole human serum; Well 4, antiserum to normal human 7S γ-globulin; Well 5 antiserum to normal human albumin.

3B. L.E. cell factor from Patient Gar. in Well 4; Wells 3 and 5 contained normal human 7S γ-globulin; Well 1, antiserum to normal whole human serum; Well 2, antiserum to normal human 7S γ-globulin.

TABLE VII

Inhibition of activity of L.E. cell factor by antiserum to normal human γ-globulin

L.E. cell formation by solution of purified L.E. cell factor	Incubated with	L.E. cell formation after incubation	γ-Globulin present after incubation (Cap. tube pptin.)*
++	Normal rabbit serum	++	++
++	Rabbit as normal human γ-globulin	0	0

* Capillary tube precipitation.

Table VII shows that the capacity to induce L.E. cell formation is completely destroyed by antiserum to normal human γ-globulin. Equal aliquots of solution of purified L.E. cell factor were mixed with equal amounts of normal rabbit serum or rabbit antiserum to normal human γ-globulin. These mixtures were incubated one hour at room temperature and overnight at 4° C. The precipitates were then spun out and the supernates analyzed for the presence of γ-globulin and for L.E. cell inducing capacity. The supernates from the L.E. cell factor incubated with normal rabbit serum retained the L.E. cell inducing capacity and γ-globulin but the L.E. cell factor and the γ-globulin were precipitated completely from the mixture with antiserum.

The L.E. cell factor also possesses the sedimentation characteristics of human 7S γ-globulin.

This is seen in Table VIII. SLE serum was separated in a sucrose gradient by density gradient zone centrifugation (24). This procedure results in a separation of the high molecular weight γ-globulin from the low molecular weight γ-globulin, the former appearing at the bottom of the tube and the latter remaining near the top. After centrifugation, the contents of the tube were divided into five fractions from top to bottom. The material in each of these fractions was analyzed for protein content, for γ-globulin by a quantitative precipitin method, and for L.E. cell activity. It will be seen that the L.E. cell activity remained in that portion of the tube where the bulk of the γ-globulin with the lower molecular weight was found, and no L.E. cell activity was found in the lower portion of the tube where the high molecular weight γ-globulin accumulated. For comparison, in the final column is shown a similar separation of serum from a patient with rheumatoid arthritis where the rheumatoid factor, which is a high molecular weight γ-globulin, was found in the bottom of the tube.

pH and temperature stability of the L.E. cell factor and its complex with nucleoprotein

Isolated L.E. cell factor was found to be stable in glycine buffers at pH 2 and pH 11 for at least 24 hours at 4° C. In isotonic NaCl, it was stable at 65° C. for at least 30 minutes; after exposure to 70° C. for 30 minutes some loss of activity occurred, while exposure to 75° C. for 30 minutes resulted in considerable though not complete loss of activity.

All experiments involving the complex of nucleoprotein and L.E. cell factor thus far reported were conducted near pH 7. An effort was therefore made to determine the stability of the complex at different pH's and at temperatures higher than 56° C. Complexes of L.E. cell factor and nucleoprotein were prepared and exposed to temperatures from 60° C. to 75° C. for 30 minutes in isotonic NaCl, or to pH 3.4, 5 and 8 in glycine buffers at 37° C. for 30 minutes. The complexes were then separated from supernates. The supernates were brought to pH 6.7 by dialysis against isotonic saline and tested for γ-globulin and ability to induce L.E. cell formation. The complexes were digested with DNase and eluted at 56° C. to recover the L.E. cell factor. Neither higher temperatures nor different pH's liberated L.E. cell factor from the complex. However, heating the complex above 60° C. or exposing it to pH 3 or below appeared to alter it in such a way as to prevent future recovery of the full amounts of γ-globulin or of active factor from the complex. Because nucleoprotein dissolves above pH 8, the influence of higher pH was not studied. It was also found that the L.E. cell factor and nucleoprotein can combine between pH 4 and pH 8. The effect of more extreme pH's upon their ability to combine was not explored.

TABLE VIII

*Distribution of L.E. cell factor, γ-globulin, total protein and rheumatoid factor in fractions of serum separated by density gradient zone centrifugation**

Fraction No.	Total protein	Immunological γ-globulin	L.E. cell formation	Sensitized sheep cell agglutination
	mg./ml.	*mg./ml.*		
1 (top)	1.2	0.1		
2	9.8	1.5	+++	0
3	8.5	3.5	++++	0
4	2.6	0.9		1/16
5 (bot.)	0.6	0.2	0	1/512

* Two-tenths ml. serum layered onto 5 cc. of sucrose in a gradient varying from 10 per cent sucrose at the top to 37 per cent sucrose at the bottom. Ultracentrifugation for 18 hours at 110,000 G. resulted in sedimentation of 19S class proteins to the bottom of the tube. Contents of tube were then collected as five separate fractions by a stabilized capillary pipette. Titers given for sheep cell agglutination refer to dilution of fraction collected.

Amount of L.E. cell factor in SLE serum

SLE is characterized by hypergammaglobulinemia. However, the L.E. cell factor in the instances tested has comprised only a small fraction of the total γ-globulin. Figure 4 shows a determination of total serum γ-globulin by the quantitative precipitin method and by zone electrophoresis before and after removal of L.E. cell factor. Only slight diminution of γ-globulin occurred despite complete removal of L.E. cell factor from a highly positive serum by absorption with cell nuclei. In one such instance, the total γ-globulin was 20.5 mg. per ml. before and 19.8 mg. per ml. after absorption.

DISCUSSION

The accumulated evidence strongly supports the view that the L.E. cell factor reacts with deoxy-

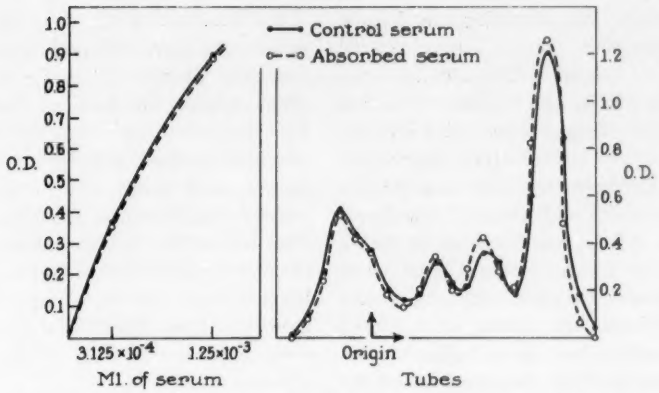

FIG. 4. EFFECT OF REMOVAL OF L.E. CELL FACTOR UPON TOTAL SERUM γ-GLOBULIN

Control serum refers to SLE serum before absorption. Absorbed serum has had L.E. cell factor entirely removed by absorption with cell nuclei. On the left is a quantitative precipitin curve using dilutions of the SLE serum and standardized rabbit antiserum to normal human γ-globulin. On the right is the serum protein pattern obtained by separation on starch in barbital buffer, ionic strength 0.1, pH 8.6. γ-Globulin is the peak to the left of the origin. Absorption caused very slight drop in γ-globulin.

ribonucleoprotein and requires both DNA and histone for reaction. This evidence includes: *1*) the two steps required for removal of the factor from the complex with nucleoprotein; *2*) the failure of the separated components of nucleoprotein or of purified DNA to react with the factor, and the partial appearance of activity on recombination of nucleoprotein components or on addition of histone to DNA; *3*) the inactivity of the complexes when long chain acids are substituted for DNA or when protamine is substituted for histone; and *4*) the apparent failure of the L.E. cell factor to displace histone from the nucleoprotein. The requirement of both DNA and histone is compatible with the results of current histochemical studies of the L.E. cell phenomenon (25).

The exact sites of combination of the L.E. cell factor and nucleoprotein remain obscure. In histochemical studies, exposure of cell nuclei to methyl green prior to absorption of the L.E. serum greatly diminished uptake of γ-globulin. Because methyl green is thought to react with the phosphate groups of DNA, it has been suggested that the L.E. cell factor also reacts with these groups. However, the inhibition of the reaction by prior exposure of nuclei to methyl green, or of nucleoprotein to histone or atabrine does not permit a conclusion on this question. It is possible that these substances interfere with the union by physical obstruction of access to the binding sites rather than by direct chemical reaction with these sites.

The biologic role of histone is unknown, and in native nucleoprotein histone may shape or hold the DNA in a particular configuration. Therefore, it is possible that in the reaction with the L.E. cell factor too, the role of the histone is to hold the DNA in proper configuration for binding and not to participate directly in the bond. However, the persistent attachment of the L.E. cell factor to the histone residue after DNase digestion of the complex argues for a direct combination between the factor and the histone.

The possible participation of the nonhistone protein of the nucleoprotein in the reaction is unsettled. Nonhistone protein comprises a minor but definite portion of the protein of nucleoprotein. It is not usually extracted by acid and it is probable that the protein which resisted extraction in these experiments was in part nonhistone protein. Since this residue absorbed a small amount of factor, reactivity of nonhistone protein cannot be excluded.

In the present experiments, the L.E. cell factors

from many different sera, including one from a patient with "apresoline disease," have reacted with nucleoprotein in an apparently identical way, all requiring both DNA and histone. This has been true regardless of reactivities which each individual serum showed toward other constituents of the nucleus. Furthermore, once nucleoprotein has been fully saturated with the L.E. cell factor from one serum, it has proved unable to absorb L.E. cell factor from another serum. Thus, while the possibility remains that L.E. cell factors may be found with different specificities, all L.E. cell factors thus far studied have been similar.

The requirement of DNA for reaction, and the apparent similarity of the L.E. cell factor in all sera studied, are at variance with the results of another study (14). The differences are probably a consequence of the methods used. In the latter study, the L.E. cell factor was not specifically identified. Instead, the absorption of γ-globulin from SLE sera onto nuclei was estimated by the fluorescent antibody method. All γ-globulin adherent to whole nuclei was assumed to be L.E. cell factor and the presence of other antinuclear antibodies was not considered. However, it is possible to explain all the results obtained on the basis of reactions of other antinuclear antibodies without inferring that the L.E. cell factor itself either differs from serum to serum, or that it does not require DNA. The existence of a group of antinuclear antibodies makes it necessary that techniques be employed which permit identification of the specific antibody in question if individual reactivities are to be studied.

The recovery of the L.E. cell factor from nucleoprotein permits a high degree of purification. The recovered solution of factor often exhibits approximately the same ability to cause formation of L.E. cells as an equivalent volume of original serum. In terms of γ-globulin concentrations, this has represented purification varying from 50- to 100-fold in different cases. No serum protein other than γ-globulin has ever appeared in the solutions of purified factor. The presence of other antinuclear factors has not been entirely excluded, though the solutions have not been able to fix complement with either pure DNA or the material extractable from nuclei with 0.1 ionic strength buffers and are presumably free of the factors reacting specifically with these materials.

The recovery of L.E. cell factor from nucleoprotein has permitted concentration of the factor and thus allowed search for the factor in sera which appear incapable of forming L.E. cells. This is conveniently done by absorbing a large amount of serum and eluting from the nucleoprotein with much smaller volumes. In preliminary experiments, L.E. cell factor has been recovered in this way from weakly positive or apparently negative sera. However, this procedure has not been done in enough cases, particularly in ones without clear clinical symptoms or complement fixation reactions of SLE, to gauge its ultimate usefulness.

Many previous experimental results appear to separate the L.E. cell factor from the factor which reacts with DNA alone. The latter factor is present far less frequently than the L.E. cell factor. It has been shown to precipitate and fix complement with pure DNA. Absorption of SLE serum with DNA alone has never appreciably reduced the capacity of the serum to form L.E. cells regardless of whether the amount of DNA used was equal to that found in equivalence point precipitates or was in excess of that amount. However, occasionally the γ-globulin recovered from equivalence point precipitates of DNA with SLE serum has shown very weak ability to form L.E. cells. Seligmann (13) has interpreted the latter finding to indicate a probable identity of the two factors.

In the present experiments, further evidence was obtained for the existence of two separate factors requiring DNA for reaction. Nucleoprotein absorbs both factors. The γ-globulin recovered from this nucleoprotein after DNase digestion alone fixes complement with pure DNA but has little if any ability to form L.E. cells. Conversely, the γ-globulin liberated by elution from the digestion residue forms L.E. cells readily but has not been capable of reaction with pure DNA. These results indicate that the DNA factor combines only with DNA of the nucleoprotein and is therefore liberated by DNase treatment alone. The L.E. cell factor combines with both DNA and histone and requires the two step procedure for liberation. Furthermore, repeated efforts to absorb the L.E. cell factor with purified DNA failed, though the factor which reacts with DNA alone was completely removed.

A likely explanation for the recovery of small amounts of L.E. cell factor from equivalence point precipitates obtained from mixtures of certain SLE sera and purified DNA would be a weak "cross reaction" between the L.E. cell factor and DNA alone. This would be consistent with the behavior of an antibody toward a substance which contains some but not all of the required combining sites. It is also possible that L.E. cell factors possess different relative affinities for DNA and histone, or that the factor which reacts with purified DNA can occasionally cause nuclear changes similar to those caused by the L.E. cell factor.

The serum factor which precipitates or fixes complement with pure DNA is present in a minority of SLE sera. Many of the SLE sera used in this study did not show these reactions with pure DNA. However, when they were absorbed with nucleoprotein and the nucleoprotein was digested with DNase, γ-globulin was liberated. Despite recovery in significant amounts, this γ-globulin was incapable of fixing complement or precipitating with DNA, or of causing L.E. cell formation. In view of the fact that such a γ-globulin has not been recovered after absorption of normal serum, it appears probable that γ-globulins with an affinity for DNA but which are incapable of precipitation or complement fixation with DNA are commonly present in SLE serum. Support for this interpretation comes from the fact that absorption of such SLE sera with pure DNA followed by precipitation of the DNA with protamine and then digestion of the DNA-protamine complex with DNase, also yields this type of γ-globulin. The reaction of this latter γ-globulin with DNA appears to be specific because it occurs above pH 7.2, which is above the isoelectric point of both γ-globulin and DNA. Therefore the net charge on both molecules would be negative, and electrostatic interactions would not be a likely cause of the reaction.

The evidence thus far accumulated suggests that the reaction of the L.E. cell factor and nucleoprotein is an immune reaction. The isolated factor has the physical and immunological properties of typical human antibody γ-globulin. It appears to have a sharply defined requirement for reaction. It is one of a group of antinuclear factors all of which are identified by standard immunological techniques. The absence of species or organ specificity exhibited by these antinuclear factors is not characteristic of many immune systems, but antibodies to pneumococcal polysaccharides possess a very broad carbohydrate reactivity (26), and antibodies to lens, brain and testes do not show species specificities (27).

The main deficiency in the evidence for an immune reaction lies in the great difficulty in obtaining analogous factors after immunization of animals with nuclear constituents (28). However, Miescher has attained some success (29). The difficulties may be due to the absence in the animals of the abnormal immune system which appears to be present in patients with SLE.

The pathogenic significance of the antinuclear factors is unknown. Considerable evidence suggests that these factors cannot enter a normal cell *in vivo* (4). They therefore appear to be by-products of an abnormal immune system rather than responsible pathogenic agents. However, exploration of this question is in its infancy.

SUMMARY

1. The lupus erythematosus (L.E.) cell factor reacts with deoxyribonucleoprotein of the cell nucleus and requires both deoxyribonucleic acid and histone for the reaction. This reaction appears to be the primary step in formation of the L.E. cell.

2. The L.E. cell factor can be isolated from its complex with nucleoprotein by digestion of the complex with deoxyribonuclease followed by elution from the residue.

3. The L.E. cell factor possesses the immunological and physical properties of typical human antibody γ-globulin.

4. The reaction of nucleoprotein with L.E. cell factor can be inhibited by prior exposure of the nucleoprotein to histone, protamine and atabrine. The mechanism of those inhibitions is not established.

5. The properties of the L.E. cell factor and the specific requirement of deoxyribonucleoprotein for reaction suggest that the reaction is immunological, and that L.E. cell factor is one of a group of antibodies to constituents of the cell nucleus which arise in systemic lupus erythematosus.

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THE MEASUREMENT OF PULMONARY DIFFUSING CAPACITY FOR CARBON MONOXIDE BY A REBREATHING METHOD*†

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The pulmonary diffusing capacity for oxygen ($D_{L_{O_2}}$) is of great physiological and clinical significance (1). Its measurement, however, is relatively complex (2). Pulmonary diffusing capacity for carbon monoxide ($D_{L_{CO}}$) which, it is usually assumed,[1] can be converted to $D_{L_{O_2}}$ from the known solubilities and molecular weights of the two gases, is, in general, more readily measured. Further, since the rate of reaction of carbon monoxide with hemoglobin has been measured (4) *in vitro* while that of oxygen has not, determination of $D_{L_{CO}}$ at two or more alveolar oxygen tensions makes possible the computation of pulmonary capillary blood volume and pulmonary membrane diffusing capacity (5–7).

The methods used for measuring $D_{L_{CO}}$ are the single breath technique of Krogh (8) as modified by Ogilvie, Forster, Blakemore and Morton (9), the steady state technique using an independent measurement of the physiological dead space (10), the steady state technique using an alveolar sample (11) and a rebreathing technique using $C^{14}O$ (12). The present paper presents a modification of the rebreathing method utilizing stable CO and continuous analysis.

METHODS

A. Experimental procedure. The apparatus shown in Figure 1 was used in these studies. The subject's vital capacity and one second vital capacity are first determined. The analyzer circuit of the apparatus is then flushed with tank oxygen. A sealed bag containing a volume of 0.3 per cent CO and 10 per cent He in air (or in oxygen)[2] equal to the subject's one second vital capacity is attached to the three-way tap, the clamps on the bag are removed and the bag and analyzer circuit mixed by the pump.[3] A bag-in-box device attached to a spirometer has been found convenient for filling the bag. The subject then expires fully through the open arm of the three-way tap, following which the tap is turned to connect him with the bag. The subject rebreathes into the bag for 30 to 45 seconds at a rate of 30 cycles per minute in time to a metronome, endeavoring to empty the bag on each inspiration. At the end of this period the tap is closed, the pump is stopped and the bag clamped and removed. Before and after the measurement of diffusing capacity the equilibrium pCO of the subject's blood is determined by a rebreathing technique (13). The bag is then analyzed for oxygen, carbon dioxide and helium by passing its contents successively through a paramagnetic oxygen analyzer,[4] an infrared CO_2 analyzer[5] and a katharometer.[6] Carbon dioxide is adsorbed before the sample is passed through the katharometer.

B. Calculations. The volume of the lung-bag system is calculated from the equation:

$$V_s = \frac{F_b V_b}{F_s},$$

where V_s = volume of the lung-bag system in milliliters STPD, V_b = volume of rebreathing bag in milliliters STPD, F_b = concentration of helium in the bag at start of rebreathing and F_s = concentration of helium in bag at end of rebreathing. V_b is obtained from the record of the spirometer attached to the bag-in-box from which the bag is filled. F_b and F_s are determined by katharometer analysis of the gas mixture used and of the bag contents at the end of rebreathing. Since CO_2 is absorbed before the gas is passed through the katharometer, this last reading is multiplied by $1 - \frac{\text{per cent } CO_2}{100}$ to obtain the final helium concentration.

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‡ This work was performed while a Fellow of the Rands Foundation.

§ Research Fellow of the Michigan Heart Association.

[1] This assumption has recently been challenged by Bates (3).

[2] Matheson Co., E. Rutherford, N. J.

[3] Dyna-Pump, Fisher Scientific Co., Pittsburgh, Pa.

[4] Model E2, Beckman Instrument Co., Pasadena, Calif.

[5] Model 16, Liston Becker Division, Beckman Instruments, Fullerton, Calif.

[6] Cambridge Instrument Co., New York, N. Y.

The subject's residual volume may readily be calculated by subtracting the volume of the bag and the dead space of the apparatus (150 ml.) from the total volume thus obtained.

The CO concentration during rebreathing is recorded by a direct writing oscillograph moving at 1 cm. per second. This record is measured at any desired interval, usually five seconds. From this measured deflection is subtracted the deflection produced by passing 5 per cent CO_2 through the CO analyzer. In two subjects the error introduced by the use of a standard correction for CO_2 was investigated by placing an infrared CO_2 analyzer in the circuit and recording simultaneously CO and CO_2 concentration in the bag. Each point of the CO record could then be corrected for the deflection due to the CO_2 present at that point by using a calibration curve for the sensitivity of the CO analyzer to CO_2. The points thus obtained were indistinguishable from those obtained by using a standard correction.

After the measured points are corrected for CO_2, the CO concentrations are read from a calibration curve and these values are corrected for the back pressure of CO in the pulmonary capillaries. The back pressure is calculated by interpolating between the initial and final equilibrium CO values obtained as described above and multiplying this interpolated value by the fraction pO_2 (bag)/pO_2 (equilibrium sample). This correction is only approximate since it assumes that all hemoglobin (Hb) is in the form O_2Hb.

After this final correction, the values for CO concentration are plotted against time on semilogarithmic paper. A line is drawn through the points beginning with the final concentration. From this line, values for CO concentration at any convenient time interval are obtained and substituted into Krogh's equation (8):

$$D = \frac{Vs}{Pb(t_2 - t_1)} \ln \frac{F_{st_1}}{F_{st_2}},$$

where F_{st_1} = concentration of CO (dry) in the lung bag system at time t_1, F_{st_2} = concentration of CO (dry) in the lung bag system at time t_2, D = D_{LCO} = apparent diffusing capacity of the lungs for CO in milliliters per millimeter Hg × minute, Pb = pressure of the dry gases in the lung-bag system, $(t_2 - t_1)$ = time of rebreathing in minutes and ln = natural logarithm.

C. Other methods. Vital capacity and one second vital capacity were measured by a rapidly moving spirometer kymograph.[7] Residual volume was measured as described above or by a closed circuit helium method (14). Maximum breathing capacity was measured with the use of a high velocity valve[8] and a 120 L. Tissot type spirometer. The single breath diffusing capacity was measured as described by Ogilvie and co-workers (9). The steady state diffusing capacity was measured by the method of Filley, MacIntosh and Wright (10); P_{ACO_2} was obtained from direct measurement of CO_2 content and pH by the use of nomogram (15). Arterial oxygen saturation was measured as described by Peters and Van Slyke (16).

RESULTS

The absorption of CO was measured continuously during a 30 to 45 second period of rebreathing 578 times in 128 subjects. In 571 studies (98.9 per cent) the disappearance of CO was apparently exponential for a period of 15 seconds or more. The exponential fall of CO began within the first five seconds of rebreathing in 236 studies (40.8 per cent), between six and 10 seconds in 229 studies (39.6 per cent), between 11 and 15 seconds in 80 instances (13.8 per cent) and between 16 and 20 seconds in 23 studies (4.0 per cent), 19 of which were of 45 seconds' duration and four of 35 seconds' duration. In four studies (0.7 per cent), all of 45 seconds, the

[7] H. S. Osborne, Havertown, Pa.

[8] Hans Rudolph, Kansas City, Mo.

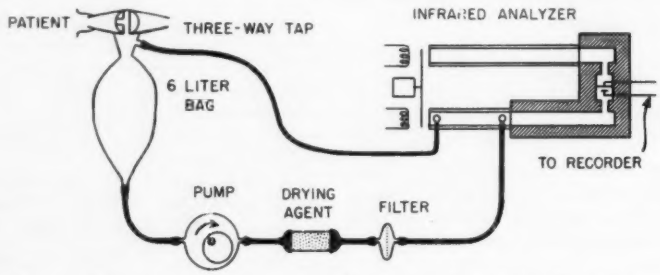

FIG. 1. DIAGRAM OF APPARATUS FOR MEASURING PULMONARY DIFFUSING CAPACITY BY REBREATHING

The pump circulates the contents of the bag through the drying agent (anhydrous $CaSO_4$), filter (Kel-F®, Liston-Becker Division, Beckman Instruments Inc.) and infrared CO analyzer (Model 15A, Beckman Instruments, Inc.) at the rate of 3 L. per minute. Volume of analyzer circuit is 150 ml. Inner diameter of openings to analyzer is one-eighth inch. Pressure at mouth during operation of pump is 0.3 cm. H_2O.

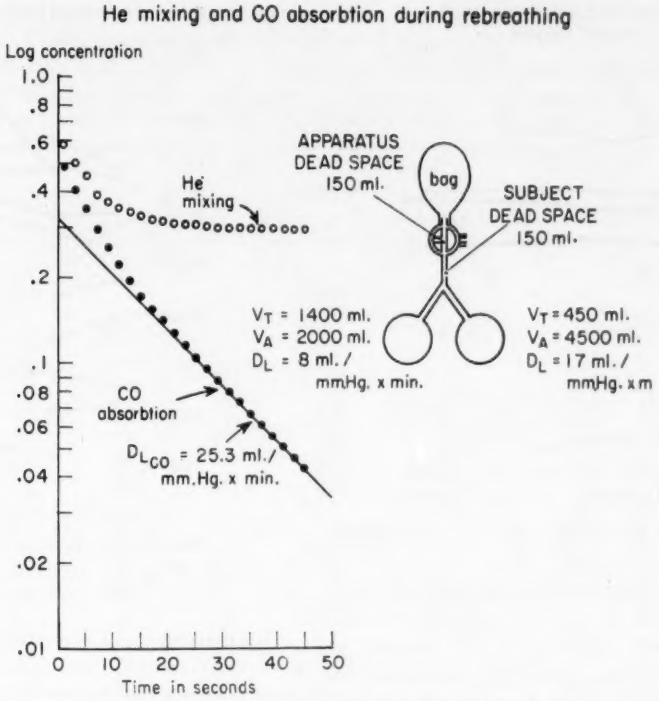

FIG. 2. HELIUM MIXING AND CARBON MONOXIDE ABSORPTION DURING REBREATHING

The concentrations of helium and CO are computed breath by breath for a 45 second period of rebreathing at 30 cycles per minute for the lung model shown at right. For explanation of abbreviations and comparison of different methods for measuring diffusing capacity in this lung model, see Table IV.

fall of CO was exponential after 21 to 25 seconds of rebreathing. When this apparently exponential decrease did not begin with the first measured point, the plot of log CO concentration prior to this time showed a gradually decreasing slope similar to that illustrated in Figure 2. This is probably due to concurrent mixing of the bag and the alveoli and diffusion from the alveoli into the blood. Those subjects in whom 10 seconds or more of rebreathing elapsed before CO concentration began to fall in an exponential manner usually had emphysema.

The continuous sampling method used in these studies was compared with discrete sampling at three points in five studies in four normal subjects (Table I). For each subject, three bags were filled to the same volume. The subject rebreathed into these bags for accurately timed periods of approximately 10, 20 and 30 seconds. The final CO concentration in each bag was measured, plotted against time and D_{LCO} calculated from the line connecting these points. As shown in Table I, this method of sampling did not give results significantly different from con-

TABLE I

*Effect of sampling method on D_{LCO}**

Subject	D_{LCO} Continuous sample	D_{LCO} Discrete sample
W. S.	28.8	28.6
E. C.	35.4	34.7
	16.1	15.4†
J. M.	28.2	26.9
R. G.	37.1	37.5
Average‡	29.13	28.63

* D_{LCO} = apparent diffusing capacity of lung for CO in milliliters per millimeter Hg × minute.
† pO_2 600 mm. Hg; other studies pO_2 100 mm. Hg.
‡ Average of five studies.

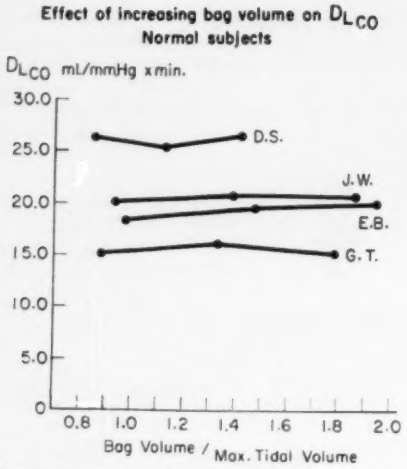

FIG. 3. EFFECT OF INCREASING BAG VOLUME ON DIFFUSING CAPACITY IN NORMAL SUBJECTS

tinuous analysis of the bag during a 30 second period.

The effect of increasing the apparatus dead space was studied in four normal subjects and five patients with emphysema by putting a greater volume in the rebreathing bag than the subject could inspire. For normal subjects the maximum tidal volume was assumed to be the one second vital capacity. Bag volumes greater than the one second vital capacity, then, represented an increase in apparatus dead space. As shown in Figure 3, such an increase did not change D_{LCO}.

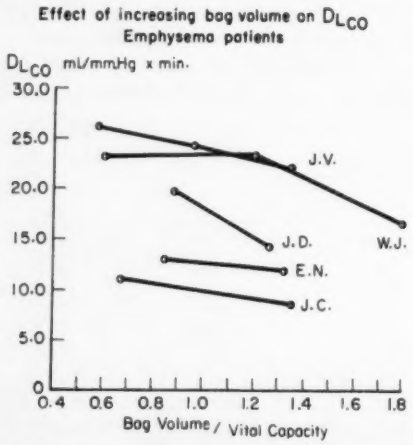

FIG. 4. EFFECT OF INCREASING BAG VOLUME ON DIFFUSING CAPACITY IN PATIENTS WITH EMPHYSEMA

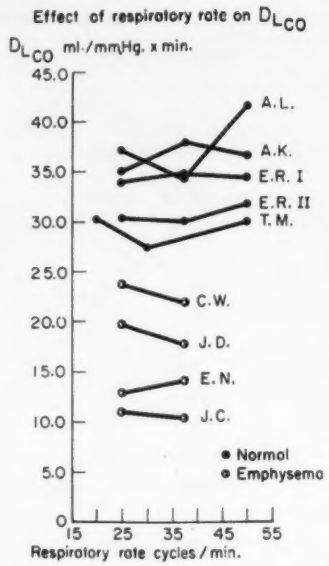

FIG. 5. EFFECT OF RATE OF REBREATHING ON DIFFUSING CAPACITY

This experiment was modified for the patients with emphysema. Previous experience with these patients had shown that when a volume of gas slightly less than the vital capacity was placed in the bag, the patient, after several

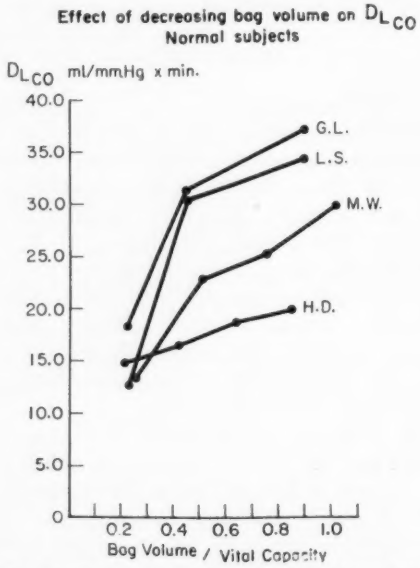

FIG. 6. EFFECT OF DECREASING BAG VOLUME ON DIFFUSING CAPACITY IN NORMAL SUBJECTS

breaths, emptied the bag completely on inspiration and yet had a tidal volume approximating his one second vital capacity. When the bag volume was appreciably less than the vital capacity, the tidal volume became very small because of air trapping during rapid expiration. Therefore, to increase the apparatus dead space, volumes of gas greater than the total vital capacity were placed in the bag. As shown in Figure 4, this led to lower values for calculated D_{LCO}.

The effect of changing rebreathing rate at the same bag volume was studied five times in four

TABLE II

Diffusing capacity of lungs in normal subjects (males)

Subject	Age	Ht.	Wt.	BSA*	VC	D_{LCO} RB	D_{LCO} SB	D_{LCO} SS
	yrs.	*in.*	*lbs.*	$M.^2$	*ml.*	*ml./mm. Hg × min.*		
H. B.	19	69	152	1.84	4,020	26.7	32.9	21.2
L. McL.	26	67	159	1.84	4,280	26.8	27.1	
M. N.	15	68	121	1.65	3,692	23.2	28.3	
W. S.	16	68	129	1.69	3,695	28.8	30.2	
J. H.	31	67	135	1.72	3,460	15.6	18.7	
J. P.	20	71	139	1.80	4,210	28.6	24.5	
C. S.	20	68	126	1.69	3,830	22.0	31.3	16.5
E. C.	31	73	178	2.06	5,025	35.4	34.8	
W. Z.	20	67	128	1.67	3,665	22.8	24.3	
J. S.	32	66	142	1.75	4,035	18.2	19.9	
R. G.	39	69	172	1.94	2,998	18.9	22.9	
C. B.	16	64	178	1.86	3,280	21.7	29.9	
H. L.	29	66	136	1.70	2,918	20.5	21.1	
T. E.	24	70	166	1.93	4,180	24.6	28.0	15.1
J. M.	25	74	186	2.11	4,800	37.5	35.0	
T. M.	25	68	168	1.91	5,419	27.5	35.5	
W. H.	47	71	151	1.88	3,760	21.9	21.8	18.5
W. S.	15	67	137	1.73	4,460	19.6	24.6	16.4
C. C.	18	70	145	1.83	4,700	33.2	32.0	
J. R.	29	68	185	1.98	4,270	23.1	28.6	
J. L.	31	73	209	2.20	4,300	26.3	32.8	
D. S.	20	71	162	1.93	4,000	26.8		
B. P.	15	70	150	1.85	4,720	30.9	25.3	
L. J.	30	73	185	2.09	4,130	34.4		
G. L.	18	65	150	1.76	4,112	37.0		
M. W.	21	70	148	1.84	3,620	29.7		
H. D.	32	72	159	1.93	4,346	19.8		
T. J.	23	67	130	1.68	3,580	25.0	26.7	
E. T.	19	70	136	1.78	3,195	18.2	17.8	
W. L.	33	74	187	2.11	4,890	40.4	41.2	
E. R.	24	72	188	2.07	5,640	32.1		
J. McG.	31	72	155	1.91	5,500	28.2		
A. Z.	23	73	205	2.18	5,150	30.5		
R. G.	25	73	180	2.06	4,935	37.1		
A. K.	23	69	158	1.91	5,000	35.1		
A. L.	24	71	160	1.92	5,104	37.1		
Average		69.62	158.20	1.88	4,157	27.3		
Diffusing capacity of lungs in normal subjects (females)								
R. T.	32	60	111	1.45	3,000	18.5	24.0	
G. T.	30	66	134	1.69	3,100	15.3		
J. W.	38	66	200	2.01	2,600	20.2		
E. B.	26	67	116	1.60	3,500	18.4		
L. B.	30	65	125	1.62	3,050	18.7		
S. H.	20	62	103	1.45	2,830	19.4		
F. A.	23	65	127	1.62	3,725	25.8		
U. B.	28	64	160	1.78	3,003	26.5		
W. G.	35	64	135	1.65	2,585	18.3		
M. R.	22	67	137	1.73	2,794	21.3		
J. C.	32	64	134	1.65	2,330	14.4		
Average		64.55	134.73	1.66	2,956	19.7		

* Body surface area.

TABLE III

Diffusing capacity of the lungs in disease

Patient	Diagnosis	Age	Sex	Ht.	Wt.	BSA	VC	RV*	1″VC	MBC	S_{aO_2}	P_{aCO_2}	D_{LCO} RB	D_{LCO} SB	D_{LCO} SS
		yrs.		*in.*	*lbs.*	*M.²*	*ml.*	*ml.*	%	*L./min.*	%	*mm. Hg*	*ml./mm. Hg × min.*		
H. K.	Emphysema	52	M	64	159	1.78	1,134	3,355	41		79	49	22.6		5.5
R. O.	Emphysema	73	M	66	131	1.68	2,630	3,325	53	53	70	42	24.1		
D. W.	Emphysema	44	M		126		1,940	2,686	60				16.7	17.5	
P. B.	Emphysema	46	M	71	170	1.97	1,980	5,075	27	33.6	95	41	29.5	36.9	
E. R.	Emphysema	52	M	67	167	1.87	3,000	2,377	33		73	69	21.8	28.8	10.0
J. U.	Emphysema	68	M	68	160	1.85	2,640	2,977	42	52	96	34	26.1	19.0	
S. P.	Emphysema	64	M	68	140	1.77	2,740	3,980	42		96	30	19.7	14.0	
W. J.	Emphysema	57	M	72	103	1.61	1,685	5,420	38				23.3	16.9	
R. J.	Emphysema	53	M				1,762	2,244	50	37	94	36	20.7	24.6	
J. Y.	Emphysema	56	M	72	111	1.66	1,820	4,800	32		90	52	19.1		
J. D.	Emphysema	65	M	66	97	1.47	2,686	3,430	30	26	91	50	18.7		
N. F.	Emphysema	59	M	74	183	2.09	2,166	4,250	38				13.4		
C. W.	Emphysema	50	M	69	125	1.71	3,219	5,150	28	47			23.7		
E. N.	Emphysema	78	M	71	123	1.72	2,131	3,258	27		95	39	14.0		
G. V.	Emphysema	63	M	66	140	1.73	2,238	3,177	29	25			13.0	9.5	
C. S.	Emphysema	39	M	74	118	1.74	3,512	3,580	26	52			19.4		
J. C.	Emphysema	74	M	64	104	1.48	1,775	3,276	43	29			11.0		
W. M.	Emphysema, bullous	70	M	65	155	1.77	1,977	3,480	43		95	38	21.6	25.8	22.3
W. F.	Emphysema, bullous	57	M	67	110	1.58	1,985	2,021	41		85	44	9.0	8.2	
D. B.	Sarcoid	23	M	74	144	1.89	1,745	584	75	80.6	88	33	8.9	13.7	
M. B.	Sarcoid	24	M	67	126	1.66	1,250	682	100	123	98	37	9.4	12.8	13.0
W. S.	Sarcoid	32	M	69	196	2.05	2,550	1,156	78		98	36	18.5	28.0	12.6
J. S.	Sarcoid	36	M	68	140	1.76	3,478	1,151	88		98	36	24.4	21.7	27.6
W. Se	Fibrosis	64	M	66	177	1.89	1,506	1,024	73	81	92	34	6.0		
E. J.	Radiation fibrosis	57	F	63	119	1.56	2,450	1,482	52	67			17.2		
A. P.	Calcification	72	M	69	137	1.76	3,435	2,850	60	63.4			7.7		
R. J.	Anemia	45	M	70	147	1.84	3,655	1,210	81				11.9	13.8	
W. Sh	Anemia	38	F	62	156	1.74	2,474	790	76				10.9	14.7	
S. S.	Polycythemia	50	M	70	163	1.92	4,080	1,747	80		98	35	24.0	37.3	13.4
G. G.	Tuberculosis	51	M	68	152	1.82	3,790	2,015	71	134	98	30	21.6	24.0	
L. D.	Obesity	42	F	68	329	2.52	1,640	988	76				20.1		
H. B.	Barrel chest	66	M	74	190	2.00	5,905	2,250	76	133			26.3		
F. H.	Hypertension	65	M	72	194	2.10	3,380	1,976	77	71	96	33	20.0	22.6	
J. Sh	Mitral stenosis	28	F	65	143	1.72	1,284	1,427					12.6	13.1	

* Abbreviations are as follows: RV, residual volume; 1″ VC, one second vital capacity as per cent of total; MBC, maximum breathing capacity; S_{aO_2}, arterial oxygen saturation; P_{aCO_2}, arterial CO_2 tension. Other abbreviations are as in previous tables. VC and MBC are measured at BTPS; other volumes are measured at STPD.

normal subjects and in four patients with emphysema (Figure 5). Variance analysis of the data for normal subjects showed no significant difference among the rates studied. Three of the four patients showed a lower value for D_{LCO} at the higher rate.

The effect of decreasing bag volume below the vital capacity was studied in four normal subjects (Figure 6). There is a marked decrease in calculated D_{LCO} when the bag volume was about 25 per cent of the vital capacity, *i.e.*, when the total volume of the lung-bag system approximated the subject's functional residual capacity.

Our data on the rebreathing D_{LCO} in 47 subjects free from heart, lung or hematologic disease are shown in Table II. Data for the single breath D_{LCO} are available in 26 of these subjects and for the steady state D_{LCO} in five. In all cases, the rebreathing D_{LCO} was measured in the seated position, the gas breathed was approximately 0.3 per cent CO in air and the bag volume was equal to the one second vital capacity. Measurements of single breath and steady state D_{LCO} were made on the same day in the seated position. The lung volume for the single breath measurement was full inspiration and for the steady state determination, functional residual capacity. The rebreathing D_{LCO} was significantly related to the height ($r = 0.624$), weight ($r = 0.506$), body surface area ($r = 0.603$) and vital capacity ($r = 0.732$). The regression equations for these relations are: $D_{LCO} = 1.265$ height (in.) $- 60.98 \pm 5.28$; $D_{LCO} = 0.1361$ weight (lbs.) $+ 4.79 \pm 5.74$; $D_{LCO} = 22.77$ body surface area

($M.^2$) − 16.11 ± 5.39 and D_{LCO} = 5.92 vital capacity (L.) + 2.22 ± 4.61.

The coefficient of variation in 35 duplicate determinations of D_{LCO} in normal subjects was 6.1 per cent.

Since these subjects were young adults, no conclusions as to the relation of D_{LCO} to age can be drawn from our data.

Data on 34 patients with pulmonary, cardiac or hematologic disease are shown in Table III. Except for the emphysematous patients, measurements of D_{LCO} were carried out as in normal subjects. In the emphysematous patients, bag volume was approximately equal to total vital capacity, rather than one second vital capacity. Additional clinical data on these patients are found in Appendix I.

In 13 duplicate determinations of D_{LCO} in patients without airway obstruction, the coefficient of variation was 5.3 per cent. In 11 duplicate determinations in patients with emphysema, the coefficient of variation was 9.2 per cent.

Figure 7 shows a comparison of the rebreathing and single breath measurements of D_{LCO} in 26 normal subjects, 10 patients without airway obstruction and 10 patients with emphysema. Also shown are comparisons of rebreathing and single breath D_{LCO} at an alveolar pO_2 of about 600 mm. Hg in four normal subjects and five patients without airway obstruction. The regression line is for the data on normal subjects and patients without airway obstruction. As discussed below, different theoretical considerations apply to the measurement of D_{LCO} by rebreathing in emphysematous patients.

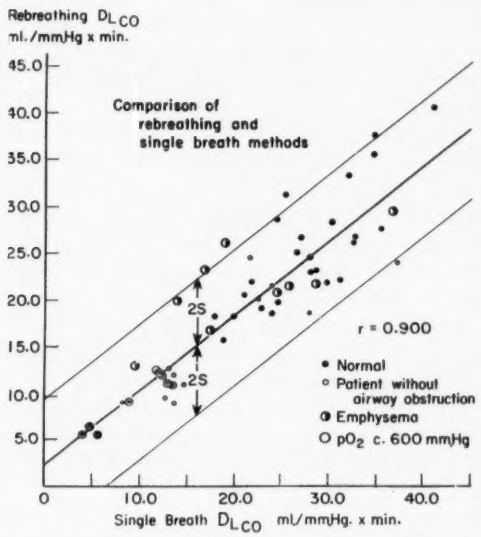

FIG. 7. COMPARISON OF MEASUREMENTS OF DIFFUSING CAPACITY BY REBREATHING AND SINGLE BREATH METHODS

The equation of the regression line is D_{LCO} (rebreathing) = 0.793 D_{LCO} (single breath) + 2.36 ± 3.68. "S" is the standard error of the estimate of this equation.

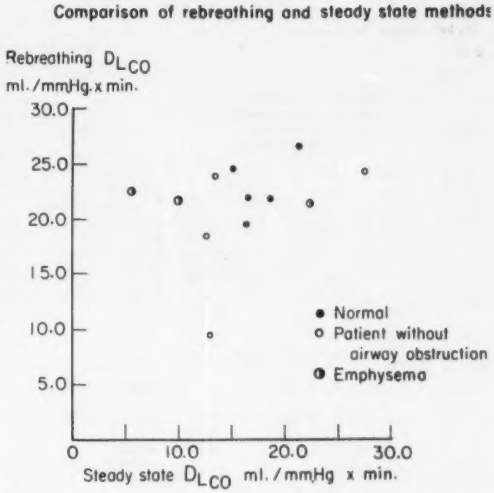

FIG. 8. COMPARISON OF MEASUREMENTS OF DIFFUSING CAPACITY BY REBREATHING AND STEADY STATE METHODS

Figure 8 shows a similar comparison of the steady state and rebreathing measurements of D_{LCO} for five normal subjects, four patients without airway obstruction and three patients with emphysema. No relation between the two measurements is evident from the rather few data available.

In Figure 9 the residual volume calculated from rebreathing data as explained above is compared with the residual volume determined in duplicate by the closed circuit helium method (14) in 28 normal subjects, 11 patients without airway obstruction and 13 patients with emphysema. The regression line shown is for the normal subjects and for the patients without airway obstruction.

DISCUSSION

Use of continuous sampling

Since we used stable rather than radioactive CO, direct comparison of our continuous sampling

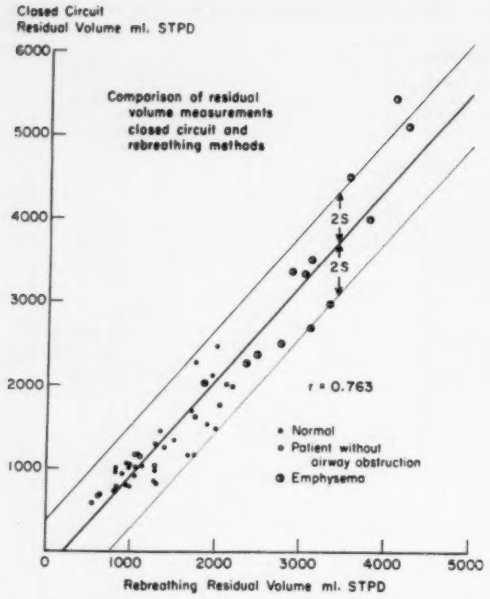

FIG. 9. COMPARISON OF RESIDUAL VOLUME MEASUREMENTS BY REBREATHING AND CLOSED CIRCUIT METHODS

The equation for the regression line is: residual volume (rebreathing) = 0.866 residual volume (closed circuit) + 228 ± 305. "S" is the standard error of the estimate of this equation.

technique with Kruhøffer's (12) technique of three discrete samples from the same rebreathing bag was not possible. However, our finding (Table I) that analysis of final CO concentration in each of three bags of the same volume gives the same rate of CO absorption as continuous sampling indicates the time lag introduced by passing gas through the analyzer circuit does not introduce any significant error into the data reported here.

Theory of carbon monoxide absorption during rebreathing

We have used Krogh's equation (8) to calculate D_{LCO} from the rate of fall of CO concentration during rebreathing. This is strictly valid only if the rate of rebreathing is infinitely fast. In this situation, the calculated D_{LCO} is independent of variations in the ratio of diffusing capacity to alveolar volume (D_L/V_A) or of diffusing capacity to alveolar ventilation ($D_L/\dot{V}_A$) among the various alveoli, since lungs, dead space and bag constitute a single well mixed system. Calculated D_{LCO} is also independent of increases or decreases in the volume of lungs, dead space or bag. The rate of rebreathing used in these studies (30 cycles per minute) is not "infinitely fast." However, it is sufficiently rapid to make the calculated D_{LCO} in normal subjects independent of increases in dead space (Figure 3). We, therefore, feel that the use of Krogh's equation (8) in such subjects is justified.

In subjects with emphysema, calculated D_{LCO} is not independent of increases in dead space (Figure 4). Rebreathing must be considered as occurring breath by breath at a finite rate. The rate of fall of CO is obtained by dividing the concentration of one breath by that of the preceding breath. The first two of a series of equations for the concentration of CO in the rebreathing bag at the end of the successive breaths are given in Appendix II. These equations are of increasing complexity and it has not been possible to obtain an equation that can be solved for D_L by dividing any such equation by that of the preceding breath.

Krogh's equation (8) can be applied to a rebreathing system that is ventilated breath by breath only if the following assumptions are made: *1*) that ratio of diffusing capacity to alveolar volume (D_L/V_A) is uniform for all alveoli. This same assumption is made in the single breath technique (17). *2*) The dead space of apparatus and subject is negligibly small. *3*) The time spent at end expiration is negligible in relation to the time spent in full inspiration.

The equation for the concentration of CO at the end of any breath then reduces to the product of a diffusion term and a mixing term. For the first two breaths these equations are:

diffusion term mixing term

$$F_{a_1} = F_I\, e^{-\frac{D_i\, Pb\, t}{V_{A_i}}} \times \sum_1^N \phi_i \frac{V_{T_i}}{V_{A_i}}$$

and

$$F_{a_2} = F_I \left(e^{-\frac{D_i\, Pb\, t}{V_{A_i}}}\right)^2 \times \sum_1^N \phi_i \frac{V_{T_i}}{V_{A_i}} \left(\frac{(V_A - V_T)_i}{V_{A_i}} + \sum_1^N \phi_i \frac{V_{T_i}}{V_{A_i}} \right),$$

TABLE IV

Calculated D_L in lung model*

Region I				Region II				Entire lung D_L			
V_{T_1}	V_{D_1}	V_{A_1}	D_{L_1}	V_{T_2}	V_{D_2}	V_{A_2}	D_{L_2}	RB	SB	SS	I + II
570	46	2,000	8	1,280	104	4,500	17	24.9	25.6	24.8	25
1,400	114	2,000	8	450	36	4,500	17	25.3	26.4	14.7	25
1,400	114	2,000	3	450	36	4,500	22	22.4	10.7	7.1	25
1,400	114	2,000	17	450	36	4,500	8	22.1	55.1	24.3	25

* Abbreviations are as follows: D_L, diffusing capacity of lung or region; V_T, tidal volume of region; V_A, volume of region at full inspiration; V_D, dead space of region. Other abbreviations are as used in previous tables. V_T, V_A and V_D are milliliters STPD; D_L is milliliters per millimeter Hg × min.

where F_I = concentration of CO in inspired gas, F_{s_1} = concentration of CO in bag at end of Breath 1, F_{s_2} = concentration of CO in bag at end of Breath 2, D_i = diffusing capacity of ith alveolus, V_{A_i} = volume of ith alveolus at full inspiration, V_{T_i} = tidal volume of ith alveolus and ϕ_i = fraction of total V_T contributed by ith alveolus. If complete mixing occurs on the first (or any subsequent) breath the mixing terms become equal. Consequently, the rate of fall of CO concentration obtained by dividing the equation for Breath 1 by that of Breath 2 is:

$$F_{s_2} = F_{s_1} e^{-\frac{D_i P_b t}{V_{A_i}}}$$

The second and third assumptions made above, however, are certainly incorrect. Further, we have continuously analyzed the helium concentration of the rebreathing circuit and found that slow mixing continues throughout a 45 second period in emphysematous patients (18).

Thus the use of Krogh's equation (8) to calculate D_{LCO} in a rebreathing system which is ventilated breath by breath at a finite rate may lead to errors in the value obtained. To evaluate the magnitude of these errors we have approached the problem empirically, using a mathematical model of the lung consisting of two regions (Figure 2). D_L/V_A and $D_L/\dot{V}_A$ are uniform within the region and may or may not be uniform between the regions (Table IV). With some labor, the concentration of an insoluble gas (helium) and a diffusible gas (CO) may be calculated at the end of each of the 23 breaths during a 45 second rebreathing period at 30 cycles per minute by expanding the equations in Appendix II. When the concentrations of CO are plotted on semilogarithmic paper, a final phase in which the disappearance of CO is apparently exponential is invariably obtained. A similar exponential phase was also obtained in a few calculations on a model lung consisting of four regions. When ventilation of the regions is uneven (Figure 2) slow mixing of helium continues during the apparently exponential disappearance of CO. The D_L calculated from this final phase of the CO curve approaches the true D_L of the model most closely when both D_L/V_A and D_L/V_A are uniform (Example 1, Table IV), but the variations from the true D_L are relatively small when D_L/D_A and $D_L/\dot{V}_A$ are not uniform as compared with the variations produced by non-uniform D_L/V_A in the single breath D_L and by non-uniform D_L/V_A in the steady state D_L.[9] From the data shown in Table IV and other similar calculations we conclude that the value of D_{LCO} calculated by a rebreathing method when the lungs are ventilated breath by breath as in emphysema, although approximate rather than exact, is not grossly in error.

Any practical consequence of this theoretical advantage, then, must rest on the demonstration that either D_L/V_A or $D_L/\dot{V}_A$ is not uniform in the lungs of the subject under study. In normal subjects ventilation is uneven to some degree (19), but Marshall (20) has concluded from fractional analysis of expired air that $D_L/\dot{V}_A$ is uniform. The evidence bearing on the uniformity of D_L/V_A in normal subjects is conflicting. Forster, Fowler, Bates and Van Lingen (21) from data on breath-holding for different periods of

[9] The theoretical values for the steady state and single breath techniques in these model lungs are calculated from the equations given by Forster, Fowler and Bates (17). The total alveolar volume during breath-holding is 6,500 ml. and the duration of breath-holding is 10 seconds. The effective tidal volume during the steady state technique is one-third that during rebreathing and the rate is 10 cycles per minute.

time thought that it was not, while Marshall (20), after repeating the same experiment, concluded that D_L/V_A is uniform. In emphysematous patients Marshall (20) found that CO concentration varied during a single expiration and concluded that D_L/V_A is non-uniform. Surprisingly, he also found that the ratio of an insoluble gas (helium) to CO during an expiration was uniform, indicating the same ratio of D_L/V_A throughout the lung. Ogilvie, Forster, Blakemore and Morton (9) also found that the ratio of He to CO was the same in an "early" as in a "late" expiratory sample in patients with emphysema.

If D_L/V_A is uniform in the lungs of normal subjects, there should be a high correlation between the rebreathing and single breath methods in these subjects. Such a correlation is found in our data (Figure 7). Further, the points for patients with emphysema scatter about the same regression line, supporting Marshall's (20) conclusion that D_L/V_A is uniform in these patients. From the available evidence, therefore, the theoretical advantage that the rebreathing D_{LCO} is independent of non-uniform D_L/V_A (infinite rate) or relatively insensitive non-uniform D_L/V_A (finite rate) does not make the rebreathing D_{LCO} more valid than the single breath D_{LCO}.

If the same type of reasoning is applied to the relation between the steady state D_{LCO} and the rebreathing D_{LCO}, one would predict a high correlation between the two methods for normal subjects and a poor correlation for emphysematous patients. These predictions are not supported by our data (Figure 8). However, no conclusions seem warranted because *1*) the data are relatively few, *2*) the lung volumes during the measurements of rebreathing and steady state D_{LCO} differ. Marshall (20) has pointed out that steady state D_{LCO} increases with lung volume and we have found a similar relation of rebreathing D_{LCO} to lung volume (Figure 6). *3*) The steady state measurements were performed at rest and the potential errors of the steady state method are greatest at rest (10).

Practical advantages of rebreathing

The following practical advantages are unique to the rebreathing method:

1) The method incorporates a continuous internal check on CO analysis. The required measurements of CO are made within seconds of each other, while the rebreathing is going on. Further, the concentration of CO should fall exponentially during a valid study. Thus, any error due to malfunction of the analyzer or leak in the system is readily detected.

2) A measurement of residual volume is obtained simultaneously with a measurement of D_{LCO}. There is a satisfactory correlation between the residual volume so obtained and a measurement using a seven minute, closed circuit, helium technique (14) (Figure 9). A corollary advantage of the rebreathing method is that calculation of the total volume of the lung-bag system by helium dilution guards against errors which may occur in the single breath method through failure of the subject to expire to his residual volume at the start of the procedure.

3) A complete measurement of D_{LCO} is very rapid. CO analysis is completed during rebreathing. The record of the CO analyzer may be measured at a minimum of three points while the rebreathing bag is analyzed for helium and carbon dioxide. All measurements and calculations can be completed in 10 minutes or less.

The following advantages of the rebreathing method are shared by either the steady state or the single breath methods:

1) No blood samples are required. *2*) Rebreathing is readily carried out by patients and untrained subjects. *3*) The method can be used in subjects with a low vital capacity. *4*) Required analysis can be done by physical methods and without highly skilled personnel.

Disadvantages of the rebreathing method

The rebreathing method has the following disadvantages:

1) Rebreathing involves considerable exercise of the respiratory muscles. We have measured the oxygen consumption[10] during rebreathing in six normal subjects by sampling continuously the oxygen concentration in the lung-bag system with a mass spectrometer.[11] The difference in

[10] Strictly, we have measured the rate of oxygen uptake by the lungs. This may not be equal to oxygen uptake by the tissues during this brief, unsteady state.

[11] Model 21-611 Consolidated Electrodynamics Corp., Pasadena, Calif.

oxygen concentration between 10 and 30 seconds was multiplied by the total volume of the system and divided by the time interval to give the data shown in Table V. For comparison, the basal oxygen consumptions of these subjects, computed from body surface area using the standards of Aub and DuBois (22) are also shown. The oxygen consumption during rebreathing is about twice the basal level. The increase in D_{LCO} corresponding to this increment in oxygen consumption apparently varies somewhat with the method. From the data collected by Forster (4), doubling the basal oxygen consumption might increase the steady state D_{LCO} by as much as 20 per cent, but would change the single breath D_{LCO} by about 5 per cent.

The oxygen cost of hyperventilation, however, is greater in emphysematous patients than in normal subjects (23). The practical importance of the fact that rebreathing is moderately severe exercise in a patient with emphysema depends on the extent to which this patient can increase his D_{LCO} on exertion. Bates, Knott and Christie (24) have reported that the steady state D_{LCO} does not increase in emphysematous patients during treadmill exercise.

2) Alveolar pO_2 falls during rebreathing. Roughton, Forster and Cander (4) have shown that the reaction rate of CO with hemoglobin is inversely proportional to pO_2; hence, the lower the alveolar pO_2 the greater D_{LCO}. With the changes in alveolar pO_2 during rebreathing, however, this effect cannot be large. In a subject with a capillary blood volume of 80 ml. and a membrane diffusing capacity of 50 ml. per mm. Hg × min., D_{LCO} would be 27.2 ml. per mm. Hg × min. at an alveolar pO_2 of 105 mm. Hg and 29.6 ml. per mm. Hg × min. at an alveolar pO_2 of 65 mm. Hg.

3) Alveolar pCO_2 rises during rebreathing. Rankin, McNeill and Forster (25) found that hypercapnia increased the single breath D_{LCO}. This effect varied with the duration of hypercapnia. When 10 per cent CO_2 was added to the mixture inspired, D_{LCO} increased 5 per cent after 10 seconds' breath-holding and 12 per cent after 50 seconds' breath-holding. When 7 per cent CO_2 was breathed for 10 minutes beforehand, D_{LCO} increased about 24 per cent. We have no data on the effect of added CO_2 on the rebreathing D_{LCO}, but the concentration of CO_2 in the rebreathing bag after 45 seconds (6 to 7 per cent) is about that to be expected after holding a breath containing 10 per cent CO_2 for 10 seconds.

TABLE V

Oxygen consumption during rebreathing

Subject	$\dot{V}_{O_2}$* Basal	$\dot{V}_{O_2}$ Rebreathing
W. G.	208	280
M. R.	221	443
J. C.	208	317
S. H.	185	369
J. McG.	260	599
J. N.	273	425
Average	225.8	405.5

* $\dot{V}_{O_2}$, oxygen consumption in milliliters STPD per minute. Basal $\dot{V}_{O_2}$ calculated on basis of body size.

Results in normal subjects

Our data, which show an average D_{LCO} of 27.3 ml. per mm. Hg × min. for 36 normal men and 19.7 ml. per mm. Hg × min. for 11 normal women, agree reasonably well with those of Kruhøffer (12) who found that the average D_{LCO} of 10 normal men, rebreathing $C^{14}O$, was 27 ml. per mm. Hg × min. and the average D_{LCO} of five normal women was 22 ml. per mm. Hg × min. Like Kruhøffer we found that D_{LCO} increases with height.

The rebreathing D_{LCO} is well correlated with the single breath D_{LCO} (Figure 7) and, like the single breath D_{LCO}, increases with body surface area, height and weight (9). Application of our regression equations and those of Ogilvie and associates (9) to subjects of the same physical parameters gives higher values for the single breath D_{LCO}, *e.g.*, for a subject of 1.73 $M.^2$ body surface area the predicted single breath D_{LCO} would be 25.8 ml. per mm. Hg × min. and the predicted rebreathing D_{LCO} would be 23.3 ml. per mm. Hg × min. Similarly, in Figure 7 values for the single breath D_{LCO} are, in general, higher than for the rebreathing D_{LCO} in the same individual. These differences, however, do not reach the level of statistical significance.

The D_{LCO} for normal females in our series is less than that for normal males. This is usually attributed to the smaller body size of women (1). Yet when our regression equations are applied to

normal females, the predicted values are higher than those actually found. Thus, the average body surface area for our female subjects was 1.66 M.², the average D_{LCO}, 19.7 ml. per mm. Hg × min. and the predicted D_{LCO}, 21.6 ml. per mm. Hg × min. Similarly, in the data of Ogilvie and co-workers (9) the average body surface area for women was 1.71 M.², the average D_{LCO}, 24.0 ml. per mm. Hg × min. and the predicted D_{LCO}, 25.4 ml. per mm. Hg × min. These differences are suggestive rather than statistically significant.

The rebreathing D_{LCO} is irregularly higher than the steady state D_{LCO} in the five normal subjects in our data (Figure 8).

Results in patients

Of our 19 patients with emphysema (Table III), in only five (N. F., E. N., J. C., G. V., W. F.) was the rebreathing D_{LCO} decreased a third or more below the predicted value based on body surface area (or weight). Thus, in 14 of these patients there was no marked impairment of diffusion. Kjerulf-Jensen and Kruhøffer (26), using a $C^{14}O$ rebreathing method, report similar findings. Only four of their 12 emphysematous patients had a D_{LCO} a third or more below the predicted value. In 10 of our emphysematous patients, D_{LCO} was also measured by the single breath technique. Only three of these (S. P., G. V. and W. F.) had a value for D_{LCO} less than two-thirds of the predicted value. Marshall (20), also using the single breath technique, found that the D_{LCO} was decreased by a third or more in only three of 11 emphysematous patients.

These findings must be contrasted with those of Ogilvie and associates (9), using the single breath technique; Bates, Knott and Christie (24), using the alveolar sample steady state technique and Donald, Renzetti, Riley and Cournand (27), using the steady state oxygen method. All of these investigators concluded that diffusion was usually impaired in emphysema.

When the same method gives conflicting results [*cf.* the results of Marshall (20) and of Ogilvie and associates (9)], the explanation presumably lies in the selection of patients. As far as the differences among the methods are concerned, we have set forth above our reasons for believing that the rebreathing D_{LCO} gives reasonably valid results in emphysematous patients despite the fact that ventilation is grossly uneven in this disease. Marshall (20) has presented evidence that the alveolar sample steady state D_{LCO} gives erroneously low values in emphysema and Kjerulf-Jensen and Kruhøffer (26) have criticized the use of the Bohr integration for determining mean capillary pO_2 in the steady state oxygen method (2).

D_{LCO} in our four patients with sarcoid varied from markedly impaired (D. B. and M. B.) to normal (J. S.). Impairment of diffusion in sarcoid was first demonstrated by Austrian and co-workers (28). Williams (29) has emphasized the frequent occurrence of normal pulmonary function in sarcoid despite widespread pulmonary involvement by X-ray.

Other authors using different techniques have reported results similar to ours in interstitial fibrosis (28), radiation fibrosis (30), anemia (31), polycythemia (31) and the "Pickwickian" syndrome (32).

SUMMARY

1. This paper describes a rebreathing method for the measurement of pulmonary diffusing capacity for carbon monoxide (D_{LCO}) using stable CO and continuous analysis.

2. The fall of CO concentration in the rebreathing bag was apparently exponential for at least 15 seconds in 571 of 578 studies. In 80.4 per cent of these studies, this exponential decrease began in the first 10 seconds of rebreathing.

3. In four normal subjects calculated D_{LCO} was independent of increases in apparatus dead space. This would be true if the rate of rebreathing were infinitely fast.

4. In patients with emphysema calculated D_{LCO} decreases as apparatus dead space is increased. This would occur if the rebreathing system were ventilated breath by breath at a finite rate.

5. In four normal subjects the rebreathing D_{LCO} decreased sharply when the total volume of the lung-bag system was decreased from near total lung capacity to approximately functional residual capacity.

6. In 47 normal subjects the rebreathing D_{LCO} was significantly correlated with height, weight, body surface area and vital capacity.

7. There is a high correlation between rebreathing and single breath measurements of

D_{LCO} in normal subjects and patients without airway obstruction. There is a less striking correlation between these two methods in patients with emphysema.

8. The residual volume determined by the rebreathing method is significantly correlated with that measured by the closed circuit helium method in both normal subjects and patients.

9. An empiric argument is presented for the approximate validity of the rebreathing method in patients with uneven ventilation and slow mixing in whom the rate of rebreathing is finite.

10. The rebreathing method is rapid, simple and gives a simultaneous measurement of D_{LCO} and residual volume.

11. The rebreathing D_{LCO} was definitely decreased (two-thirds or less the predicted value) in only five of 19 patients with emphysema.

APPENDIX I

The diagnosis of emphysema in patients H. K. through J. C. (Table III) was based on: *1*) a low one second vital capacity and/or maximum breathing capacity, *2*) an increased residual volume and *3*) slight or no improvement in these tests after bronchodilators. All patients were dyspneic and their physical findings and X-rays were compatible with chronic obstructive emphysema. Of these patients, complicating diseases were present in the following: S. P. (aneurysm of the abdominal aorta, carotid artery thrombosis), J. D. (peptic ulcer), N. F. (rheumatoid arthritis), E. N. (myocardial infarct), C. S. (peptic ulcer) and J. C. ("coin lesion" right lung). The chest X-ray of W. M. showed a large bulla in the left lower lung and the chest X-ray of W. F. showed a large bulla in the left upper lung field. Patients D. B. through J. S. had widespread, soft, patchy densities on X-ray of the chest. In each, either a scalene node (D. B., J. S.) or a lung biopsy (W. S., M. B.) was compatible with sarcoid histologically. D. B. and M. B. were dyspneic while W. S. and J. S. were working full time. W. Se. had relentlessly progressive dyspnea for two years. Chest X-ray revealed diffuse fibrotic infiltration. E. J. received Co^{60} therapy after radical removal of carcinoma of the left breast. At the time of these studies she had a severe cough and a soft tissue density was present in the apical and infraclavicular portions of the left lung field. A. P. had widespread miliary calcific densities on chest X-ray. He was severely dyspneic. Histoplasmin and tuberculin skin tests were positive, but cultures for tuberculosis were negative. R. J. was diabetic and had a hemolytic anemia of unknown cause. His hemoglobin at the time of study was 8.0 Gm. per cent. W. Sh. was anemic because of bleeding from a small bowel tumor. Her hemoglobin was 6.9 Gm. per cent. The hemoglobin in S. S. was 18.6 Gm. per cent. G. G. had exudative lesions in both upper lung fields. Sputum smear was positive for tuberculosis. L. D. was somnolent, dyspneic, weak and grossly obese. H. B. had a markedly increased anteroposterior diameter of the chest with dorsal kyphosis. The lung fields showed increased radiolucency. F. H. was hypertensive (190/90 mm. Hg) and had been in congestive heart failure twice before. He had a lesion of the left mid-lung field that proved to be carcinoma at thoracotomy. J. Sh. had the classic murmur of mitral stenosis. She had several episodes of acute pulmonary edema.

APPENDIX II

During the first breath of a rebreathing experiment the tidal volume (V_T) of the subject flushes the dead space (V_D) with the concentration of CO in the bag (F_I) and brings $F_I(V_T - V_D)_i$ ml. of CO to the ith alveolus. This volume of CO is contained in the end inspiratory volume of the alveolus (V_{A_i}). Hence, the concentration of CO in the ith alveolus at the end of inspiration, which is assumed to be instantaneous, is $F_I \frac{(V_T - V_D)_i}{V_{A_i}}$. If full inspiration lasts t minutes, this concentration falls because the ith alveolus has a certain diffusing capacity for CO (D_i). The rate of fall of CO concentration is the power of e (base of the natural logarithm) $e^{-\frac{D_i Pb\, t}{V_{A_i}}}$, where Pb is the pressure of dry gas in the alveoli. On expiration, which is also assumed to be instantaneous, the ith alveolus contributes a fraction (ϕ_i) of the total ($V_T - V_D$). The concentration of CO in the rebreathing bag at end expiration (F_{e_1}) is then a mixture of the contribution of the apparatus dead space (V_{D_a}), the subject's dead space and what was expired from the alveoli. Thus,

$$F_{e_1} = F_I\left(\frac{V_D + V_{D_a}}{V_T + V_{D_a}} + \frac{V_T - V_D}{V_T + V_{D_a}} \sum_1^N \phi_i \frac{(V_T - V_D)_i}{V_{A_i}} e^{-\frac{Pb\, t\, D_i}{V_{A_i}}}\right).$$

During full expiration, which also lasts t minutes, the concentration of CO remaining in the ith alveolus falls by the power $e^{-\frac{D_i Pb\, t}{(V_A - V_T)_i}}$. On the second breath the concentration of CO in the dead space is F_{e_1}. The ith alveolus receives first a volume (V_{D_i}) of the mixed alveolar gas in the dead space at the end of expiration (the "alveolar" part of the equation for F_{e_1}) and then a volume $(V_T - V_D)_i$ of the gas in the bag. Both of these moieties are mixed with the gas remaining in the alveoli during expiration to give the concentration of CO at the end of the second inspiration. During a full inspiration of t minutes, CO again diffuses out of the alveolus as explained above. The concentration of CO in the bag at the end of the second expiration (F_{e_2}) is:

$$F_{e_2} = F_{e_1}\frac{V_D + V_{D_a}}{V_T + V_{D_a}} + \frac{V_T - V_D}{V_T + V_{D_a}} \sum_1^N \phi_i \left[F_I\left(\frac{V_{D_i}}{V_{A_i}} \sum_1^N \phi_i \frac{(V_T - V_D)_i}{V_{A_i}} e^{-\frac{D_i Pb\, t}{V_{A_i}}}\right) + F_I\left(\frac{(V_A - V_T)_i}{V_{A_i}} \frac{(V_T - V_D)_i}{V_{A_i}} e^{-\frac{D_i Pb\, t}{V_{A_i}}} e^{-\frac{D_i Pb\, t}{(V_A - V_T)_i}}\right) + F_{e_1} \frac{(V_T - V_D)_i}{V_{A_i}}\right] e^{-\frac{D_i Pb\, t}{V_{A_i}}}.$$

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VELOCITY OF BLOOD FLOW AND STROKE VOLUME OBTAINED FROM THE PRESSURE PULSE *

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There are several methods available for obtaining the velocity of blood flow from the aorta (1–5). The bristle flowmeter and the electromagnetic flowmeter are two such methods which are satisfactory in some circumstances; however, both require an open chest preparation and isolation of the aorta. More recently it has been suggested that if suitable restrictions are imposed on the Navier-Stokes equations, the axial aortic pressure gradient, $\partial p/\partial x$, can be related approximately to the instantaneous blood velocity, u, by the equation:

$$-\frac{\partial p}{\partial x} = \rho \frac{du}{dt} + au, \qquad 1)$$

where x is the axial coordinate of the tube, t is time, ρ is the blood density, and "a" is a "blood friction" constant. This equation can be instantaneously and continuously solved for u, the blood velocity, by simple analog computer technics if the pressure gradient, $\partial p/\partial x$, is known (6, 7). The pressure gradient can be estimated by measuring the difference in lateral pressure between two points several centimeters apart along the aorta. This pressure difference may be obtained with a suitable double lumen catheter. Consequently, this method has a great advantage over previously described technics since it may be used in the intact animal or man.

This method has the great disadvantage, however, of requiring a large catheter (usually necessitating the sacrifice of an artery) and two exactly balanced manometers. Therefore, a modification permitting the use of a single catheter and a single manometer would be advantageous. It will be the purpose of this report to suggest a further restricting assumption which permits use of a single lumen and to present data that lend support to the validity of this assumption.

THEORY

If a pressure wave travels undistorted and without reflection down an elastic tube, then the instantaneous pressure gradient, $\partial p/\partial x$, is related to the time derivative of pressure, $\partial p/\partial t$, at a point by

$$-\frac{\partial p}{\partial x} = \frac{1}{s}\frac{\partial p}{\partial t}, \qquad 2)$$

where s is the pressure pulse wave velocity down the tube. Substituting Equation 2 into Equation 1 gives:

$$\frac{\partial p}{\partial t} = \rho s \frac{du}{dt} + asu. \qquad 3)$$

This equation may be instantaneously and continuously solved for the velocity, u, by the same computer technics previously mentioned if $\partial p/\partial t$ is measured.

METHODS

Eight dogs were anesthetized with Nembutal® and tracheotomized for positive pressure artificial respiration. The left chest was opened. A large cannula connected to a reservoir of blood was placed in the left atrium so that the atrial pressure could be varied as desired.

Pressures from the ascending aorta were obtained through a PE 250 Odman Ledin catheter attached to a Statham P23D pressure transducer. An eight channel Electronics for Medicine [1] recorder was used. The frequency response of the system consisting of the catheter, pressure transducer and strain gauge amplifier was checked and determined to be flat to 50 cycles per second. The output from the strain gauge amplifier was fed into another amplifier through an RC differentiator [1] so as to obtain the first time derivative of aortic pressure. This differentiator is reliable up to 80 cycles per second. The signal from the amplifier carrying the first derivative of the aortic pressure pulse was led through the analog computer described by Fry, Mallos, Casper and Noble (6, 7). This computer has an adustment which provides for variations in the frictional and inertial factors of the

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[1] Made by Electronics for Medicine, Inc., White Plains, New York.

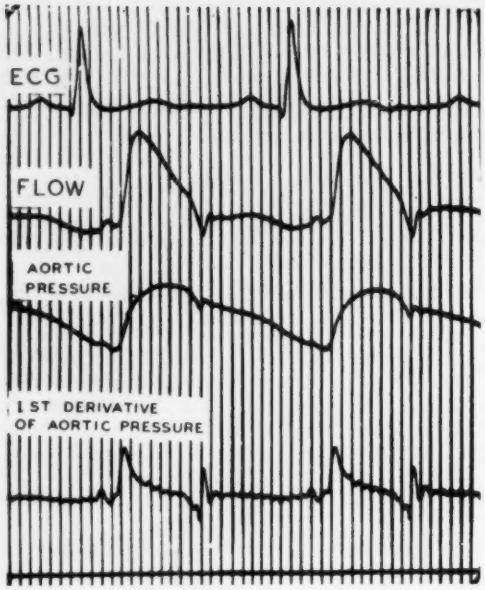

FIG. 1. A REPRESENTATIVE TRACE SHOWING THE ECG, THE BLOOD VELOCITY CURVE, THE AORTIC PRESSURE PULSE AND THE FIRST TIME DERIVATIVE OF THE AORTIC PRESSURE PULSE

The paper speed is 100 mm. per second and the time lines are 0.04 second.

Navier-Stokes equation. The resulting signal is led into a third amplifier and recorded as the velocity curve.

The frictional and inertial factor computer adjustment was set so that the velocity curve obtained was on the baseline during the last one-third to one-half of diastole. This setting was made by eye and is necessarily somewhat arbitrary. However, once the setting was made on each dog, no further changes were made during the course of the experiment.

In each of the eight dogs used, the cardiac output was varied by changing the atrial reservoir level. In six of the dogs, the cardiovascular status was further varied by infusions of first epinephrine and then methoxamine.

Multiple dye dilution cardiac output determinations were made on each dog, using Cardio-Green [2] and the Ensco Oximeter.[3] While the dye dilution curve was being obtained on one recorder, simultaneous aortic pressure pulse, first time derivative of the aortic pressure pulse, aortic blood velocity and electrocardiogram were obtained on a second recorder (Figure 1). These simultaneous determinations were made under widely varying conditions of heart rate, cardiac output and blood pressure.

[2] Supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md.

[3] Manufactured by ENSCO Engineering Specialty Co., P.O. Box 19, Sugar House Station, Salt Lake City, Utah.

RESULTS

The data were calculated as follows: The minute systolic area under each velocity curve was determined planimetrically. This was done by measuring from five to 15 consecutive complexes, determining the average and multiplying by the heart rate. A constant was determined for each dog to convert the area under the velocity curve into cubic centimeters of blood by dividing the second dye dilution cardiac output by the cor-

TABLE I

Comparison of cardiac outputs estimated by dye dilution and blood velocity methods *

	I	II		I	II
	866	1,037		886	902
Dog	1,477	1,477		1,040	1,040
No. 1	1,970	1,799		1,770	1,376
	2,166	1,973		2,180	1,792
			Dog	1,600	1,452
	1,180	1,119	No. 6	1,150	1,074
	1,668	1,668		1,815	1,274
Dog	2,000	2,074		1,579	1,083
No. 2	3,030	2,465		2,213	2,044
	1,450	1,524		1,310	1,103
	436	443		1,569	1,637
Dog	596	596		2,140	2,140
No. 3	827	737		3,363	3,231
				2,900	2,839
	560	497	Dog	2,320	2,388
	800	800	No. 7	2,085	1,867
	1,230	1,168		3,063	2,259
	1,330	1,361		3,399	2,819
	1,070	1,092		2,933	2,231
	870	1,107			
Dog	1,010	1,361		1,886	1,900
No. 4	1,420	1,527		1,877	1,877
	1,290	1,135		1,445	1,472
	420	373		1,127	1,077
	446	377	Dog	1,300	1,334
	720	558	No. 8	1,277	1,422
	400	408		2,071	1,663
				1,369	1,142
	1,380	1,613		636	810
	1,970	1,970			
	2,680	2,418			
	2,380	2,207			
	2,440	2,150			
	1,980	1,631			
Dog	1,820	1,386			
No. 5	1,950	1,660			
	1,805	1,877			
	2,300	2,291			
	2,820	2,859			
	2,360	2,235			
	1,025	1,094			

* Column I is the cardiac output in cubic centimeters per minute as determined by dye dilution. Column II is the corresponding area under the blood velocity curve adjusted to cubic centimeters per minute with a constant determined by dividing the second cardiac output in each dog experiment by the corresponding area under the velocity curve.

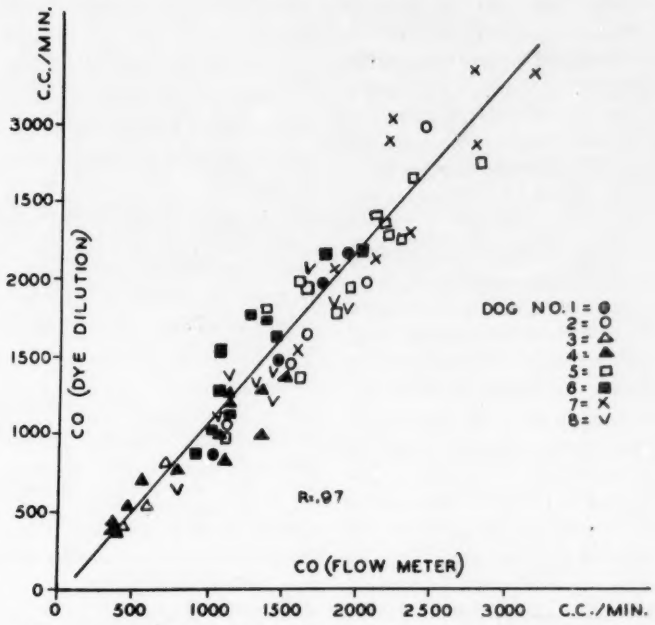

FIG. 2. COMPARISON OF CARDIAC OUTPUTS BY DYE DILUTION AND BLOOD VELOCITY METHODS

The ordinate is the cardiac output as determined by dye dilution. The abscissa is the corresponding area under the aortic velocity curve converted to cubic centimeters per minute by a constant determined by dividing the second cardiac output in each dog experiment by the corresponding area under the velocity curve. The correlation coefficient is 0.97. The average deviation is 10 per cent and the maximum deviation is 35 per cent. Individual correlation coefficients are from dogs 1 to 8: R = 0.99, 0.96, 0.99, 0.94, 0.92, 0.91, 0.85 and 0.92, respectively, $(y = -45 + 1.1x)$.

responding minute systolic area. The second determination in each dog was used because the left atrial pressure was near normal at this time. The results are presented in Table I, Column II, and graphically in Figure 2. A correlation coefficient of 0.97 is obtained.

DISCUSSION

In this study, the data demonstrate the close correlation of the area under the flow curve to stroke output. The velocity curve obtained is, from mathematical considerations, proportional to blood velocity (centimeters per second). This is proportional to flow (cubic centimeters per second) provided the size of the vessel does not change appreciably. This would appear to be the case because no significant correlation was found between the mean ejection blood pressure and the ratio of cardiac output by the flowmeter method to the cardiac output by the dye method. If changes in aortic size were an important cause of the scatter noted in Figure 2, this method of analysis should have detected it. The maximum variation of mean ejection pressure was found in Dog No. 4 and was from 50 to 249 mm. Hg.

No evidence of the validity of the form of the velocity curve was obtained. However, a simultaneous comparison with a bristle flowmeter has been found to give curves of similar contour. Fry, using his method, has demonstrated his velocity curves to have the same contour as those obtained with an electromagnetic flowmeter (7).

In a previous communication from this laboratory, it has been pointed out that the spatial aortic pressure differential and the first time derivative

of pressure are similar in timing and contour. This relationship lends support to the basic assumption in this method that there is relatively little reflected wave energy in the ascending aorta.

From the mathematical analysis, it is apparent that variations in the rate of pulse transmission (S) will affect the recorded blood velocity. From the data presented, this variation appears to be insignificant within the conditions of the experiment.

The primary advantage of this method of application of the Navier-Stokes equation is that a single lumen catheter is used. This permits the use of a single catheter-manometer system which has a good frequency response. To obtain comparable results with the two-lumen method, both manometer systems must be set at the same sensitivity and have the same frequency response. These conditions are difficult to obtain. However, it must be pointed out that the presence of significant reflected pressure waves will theoretically distort the recorded velocity curve using the time derivative. This objection does not apply to the use of the spatial pressure differential. To illustrate, if pressure is measured at a single point, the computer is fed the same signal whether a given component of the pressure pulse is traveling toward or away from the aortic valve, but with the two-manometer system the signal fed the computer is opposite in phase if a given component is traveling upstream as a reflected wave. The calibration method used for stroke output (a comparison with a dye dilution cardiac output) is advantageous in that the size of the aorta is not a factor.

The method described appears to be a relatively simple method of obtaining beat-by-beat stroke volume and blood velocity in any situation where an accurate pressure pulse can be recorded. Obtaining pressure pulses from the human ascending aorta is practical using a small radiopaque catheter passed retrograde from the brachial artery by the Seldinger percutaneous technic (8). Since a small catheter is suitable, stroke volume can be obtained without permanent damage to the brachial artery.

SUMMARY

A method for using a single lumen catheter in recording a continuous blood velocity in the aorta is described. The system is based on the Navier-Stokes equation and uses an electronic computer. The cardiac output as determined by the area under the velocity curves is compared with cardiac outputs determined simultaneously with the dye dilution technic. A correlation coefficient of 0.97 is obtained.

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FLUORESCENT ANTIGLOBULIN STUDIES IN LEUKOPENIC AND RELATED DISORDERS *

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Increasing interest in the search for antileukocyte factors in clinical syndromes characterized by leukopenia has been evident in recent years (1–6). The demonstration of what appear to be auto-antibodies in idiopathic thrombocytopenic purpura (7–9) and acquired hemolytic anemia (10–12) has, by analogy, directed attention to the possibility of a similar mechanism operating in certain leukopenic conditions (1–6, 13). Similarly, the effects of drug sensitivity have been recognized to affect any or all of the three formed elements of the peripheral blood (14–20). Evidence has also been presented that in patients requiring multiple transfusions, powerful leukocyte antibodies may be produced and give rise to undue reactions (21–23). Finally, the reported occurrence of anti-leukocyte substances in patients with aleukemic leukemia and Hodgkin's disease presents another area for further investigation (2, 24–27).

The lack of a simple, reproducible, reliable technique for the detection of leukocyte antibodies still constitutes a limiting factor in this branch of immunohematology (3, 25, 28, 29). With the development of fluorescein-labeled antibodies (30), a new tool has become available for the study of immune reactions. This paper deals with the application of this method in the search for anti-leukocyte factors in several disorders, with special emphasis on rheumatoid arthritis, Felty's syndrome and systemic lupus erythematosus (SLE). A preliminary report has been presented (31).

MATERIALS AND METHODS

Blood smears. Capillary blood from normal individuals of varying ABO and Rh blood types was used. Smears prepared by the slide method were immediately fixed in 95 per cent ethanol for 45 minutes, rinsed in distilled water and dried before using.

Human sera. Sera from 112 individuals were obtained. When not tested within 24 hours from the time they were drawn, they were stored at −20° C. until used. Twenty laboratory workers served as normal controls. All the patients studied were examined by at least one of

TABLE I

Results of test on sera of 112 individuals studied

Clinical diagnosis	Total no. of cases	Nuclear leukocyte fluorescence	
		Positive	Negative
Systemic lupus erythematosus	15	15	0
Rheumatoid arthritis			
Felty's syndrome	10	10	0
Uncomplicated	13	2	11
Periarteritis nodosa	1	0	1
Serum sickness	1	0	1
Erythema nodosum	1	0	1
Rheumatic fever and chorea	3	0	3
Discoid lupus	2	0	2
Hyperglobulinemia			
Laennec's cirrhosis	3	0	3
Bronchogenic carcinoma	1	0	1
Multiple myeloma	3	0	3
Asthma (on steroids for 3 years)	3	0	3
Multiple transfusions	4	0	4
Idiopathic thrombocytopenic purpura	5	0	5
Granulocytic leukemia			
Aleukemic	3	0	3
Leukemic	7	0	7
Lymphocytic leukemia			
Aleukemic	3	0	3
Leukemic	2	0	2
Polycythemia vera	8	0	8
Hodgkin's disease	4	0	4
Normal laboratory personnel	20	0	20
Totals	112	27	85

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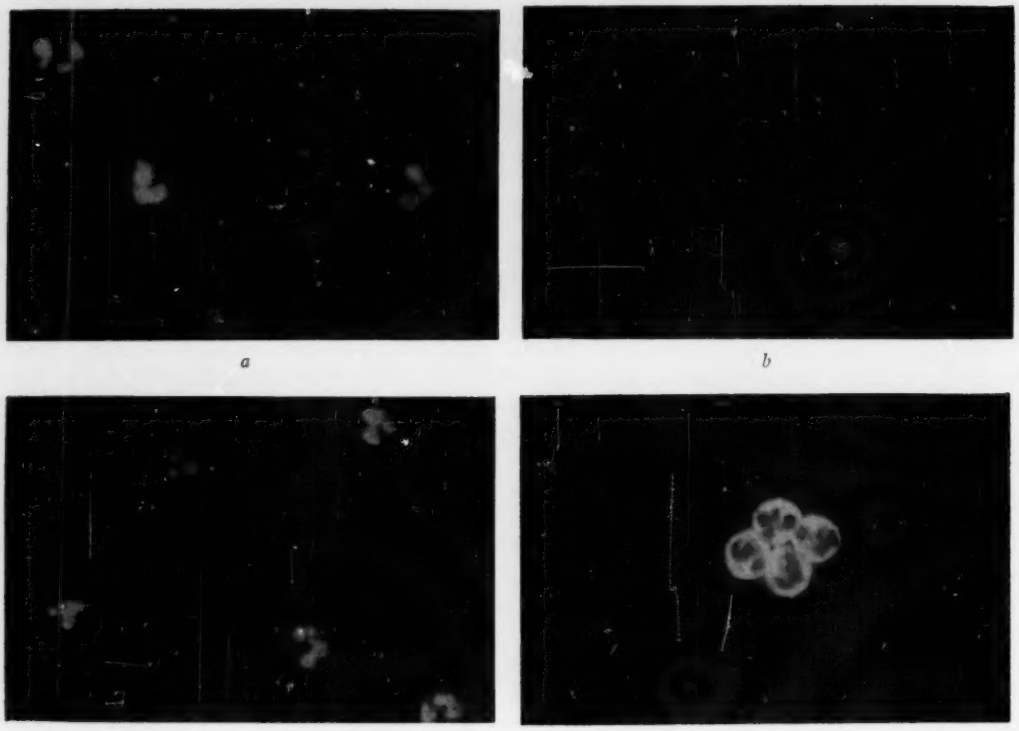

FIG. 1. PHOTOMICROGRAPHS OF POLYMORPHONUCLEAR LEUKOCYTES FROM NORMAL BLOOD SMEARS TREATED WITH HUMAN SERA AND SUPERIMPOSED WITH FLUORESCEIN-LABELED RABBIT ANTIHUMAN GLOBULIN (COOMBS') SERUM

Human sera from: *a*) systemic lupus erythematosus; *b*) normal control; *c*) Felty's syndrome; *d*) normal control exposed to antiglobulin *not* absorbed with rabbit bone marrow. Note nonspecific cytoplasmic fluorescence.

us, except where otherwise indicated. The different disease states investigated are listed in Table I.

Preparation of fluorescent antihuman globulin (Coombs') serum. Human γ-globulin (Cohn Fraction II) in Freund's adjuvant (32) was injected intramuscularly into 3 Kg. male albino rabbits. The dosage schedule was 50 mg. of γ-globulin on four successive days of each week for four weeks with a repeat series after a two week interval. The animals were sacrificed seven days following the last injection. The sera with precipitin titers varying from 1:40,000 to 1:80,000 were pooled and stored at −20° C.

The fluorescein [1] conjugate was prepared by the method described by Coons and Kaplan (30) with the following modification. The rabbit serum was precipitated twice with a half saturated ammonium sulfate solution, resuspended in 0.15 M NaCl and dialyzed against a solution of 0.15 M NaCl containing 0.01 M phosphate until no sulfate was detectable (usually four to six days) before conjugation with fluorescein isocyanate. The precipitation and dialysis were carried out at 4° C.

The labeled conjugate was stored in 5 to 10 ml. aliquots at −20° C. and was absorbed twice with acetone-dried rabbit bone marrow (33, 34) before use.

Testing procedure. A few drops of the human serum to be tested were placed on a thin portion of the slide in direct contact with the fixed normal blood smear and allowed to stand at room temperature in a moist chamber for one hour. The slide was then washed in running tap water for at least 30 minutes and air dried. The labeled rabbit antihuman globulin serum (Coombs' serum) was then superimposed on the test area of the smear for one hour under the same conditions. Vigorous washing for one hour or more was necessary to remove nonspecific labeling, and an even clearer differentiation was obtained if the preparations were allowed to stand 24 hours before making the observations by ultraviolet microscopy.

[1] The fluorescein amine was kindly supplied by Dr. Leland Pence, of Difco Laboratories.

Readings. All slides were read "blind" by each one of the authors. Early in the study, as many as five separate readings were done by each observer on different days, and some sera were tested on multiple occasions against smears from six or more individuals of different blood groups. It soon became apparent, however, that consistent results were being regularly obtained, and as our experience increased, two readings apiece were considered sufficient. For the sake of uniformity, only smears from O Rh-negative normals were used in the latter part of the study, although definite, naturally occurring leukocyte isoantibodies could not be demonstrated and no specific relationship to erythrocyte antigens was observed (1, 23, 35–37). An important aspect of the interpretation rests upon having a known positive serum and a known negative serum included in each series of slides stained. The presence or absence of the apple-green (specific) fluorescence constituted a positive or negative reading.

Microscopy and photographic equipment. A Leitz Ortholux microscope with a cardioid dark field condenser was used, with a 150 watt Philips mercury vapor lamp providing the ultraviolet light source. Thirty-five mm. daylight super Anscochrome® film was used for recording the slides.

Lupus erythematosus (L.E.) cell preparations and sheep cell hemagglutination titers. L.E. cell preparations were performed by the method of Magath and Winkle (38). The sheep cell hemagglutination titers were carried out as described by Craig, Kerby and Persons (39).

In vivo studies. Whole blood was collected in vacuum bottles containing acid citrate dextrose (ACD) solution and, after centrifuging, the plasma was transferred to vacuum containers under aseptic conditions and stored at $-20°$ C. until ready for use. The recipients were ABO and Rh-compatible patients with far advanced neoplasms not obviously involving the bone marrow and with normal peripheral leukocyte counts. In each case the recipient was given first the plasma from a leukopenic subject and, several days later, a similar amount of normal plasma under identical conditions.

RESULTS

Nonspecificity of unabsorbed fluorescent antihuman globulin (Coombs') serum

The use of labeled antihuman globulin serum which had not been absorbed with acetone-dried rabbit bone marrow caused cytoplasmic fluorescence of leukocytes previously exposed to normal serum (Figure 1*d*). This made the differentiation of positive and negative smears extremely difficult or impossible. After absorption with rabbit bone marrow, however, this nonspecific cytoplasmic labeling diminished markedly (Figure 1*b*), allowing recognition of unequivocal differences between cell nuclei coated with positive and negative sera (Figures 1*a*, 1*b*, and 1*c*).

Specificity of absorbed antihuman globulin (Coombs') serum

To demonstrate the specificity of the labeled conjugate for human globulin, nonfluorescent rabbit antihuman globulin serum was superimposed on blood smears previously exposed to known positive sera from patients with SLE or Felty's syndrome. After subsequent exposure to labeled conjugate, the nuclei showed no fluorescence.

Systemic lupus erythematosus, rheumatoid arthritis and allied disorders

Fifteen patients with SLE were studied (Table II). In each case a strongly positive nuclear fluorescence was obtained by the technique described (Figure 1*a*). No difference was observed when smears of several individuals of various blood groups were used. All except one had positive L.E. cell tests at the time the fluorescein test was performed. One patient (C.S.) was of special interest because of a positive nuclear fluorescence reading with absence of demonstrable L.E. cells. Her clinical picture was quite compatible with SLE and on subsequent examination, two months later, several positive L.E. preparations were obtained with her serum.

The sera of 23 patients with the clinical picture of rheumatoid arthritis were obtained (Table II). In 10 of these a clinical diagnosis of Felty's syndrome was made and, for the purposes of the discussion, this group will be considered separately. In general this "label" was used when the classic triad of leukopenia, splenomegaly and rheumatoid arthritis was present without serological or other clinical manifestations of SLE. The following exceptions or variations to this picture were present. Four patients with Felty's syndrome were studied post splenectomy and, though previously leukopenic, their total leukocyte counts were normal at this time. Splenomegaly was, or had been, present in all but two cases; one of these (I.B.) was the only patient in this group with a positive L.E. cell test. She had a documented history of rheumatoid arthritis for 26 years with classical, severe joint changes, and at no time had any evidence of serositis, nephritis, carditis or

TABLE II

Patients with systemic lupus erythematosus, Felty's syndrome and rheumatoid arthritis

Patient	Age and sex	Clinical diagnosis	Splenomegaly	WBC $\times 10^3$	Serum proteins A/G	Sheep cell titers	L.E. prep.	Nuclear fluor.
					Gm./100 ml.			
1. A. T.	39F	SLE	Not Palp.	5.8	3.4/5.5	1:20	+	+
2. S. C.	31F	SLE	Not Palp.	5.0	4.5/3.4	Neg.	+	+
3. C. S.	52F	SLE	Not Palp.	3.8	4.3/5.5	1:320	Neg.*	+
4. H. N.	33F	SLE	Not Palp.	5.8	3.0/4.4	1:200	+	+
5. I. L.	58F	SLE	Not Palp.	3.3	2.6/3.4	1:1600	+	+
6. B. M.	15F	SLE	Not Palp.	5.4	1.9/5.6	Neg.	+	+
7. L. T.	46F	SLE	Not Palp.	5.5	4.5/3.6	Neg.	+	+
8. D. Mi.	22F	SLE	Not Palp.	6.0	4.3/3.6	1:80	+	+
9. D. Me.	32F	SLE	Not Palp.	14.0	3.4/5.0	Neg.	+	+
10. R. D.	44F	SLE	Not Palp.	3.1	2.7/3.9		+	+
11. J. D.	18F	SLE	Not Palp.	3.4	1.2/2.3		+	+
12. M. G.	15F	SLE	Not Palp.	4.5	3.1/2.3	1:20	+	+
13. D. B.	31M	SLE	3 cm.	7.1	3.8/5.3	1:20	+	+
14. G. N.	62M	SLE	Not Palp.	4.9	2.8/4.6	1:80	+	+
15. B. V.	25F	SLE	Not Palp.	7.5	4.3/4.3	1:20	+	+
1. I. B.	59F	Felty's	Not Palp.	3.0	3.0/3.2	1:400	+	+
2. R. S.	65M	Felty's	10 cm.	1.0	2.8/4.1	1:80	Neg.	+
3. B. H.	79F	Felty's	4 cm.	1.2	3.7/3.9	Neg.	Neg.	+
4. E. B.	66M	Felty's	Not Palp.	2.8	4.7/3.4	1:1280	Neg.	+
5. K. F.	70F	Felty's	By X-ray	0.8	3.2/4.0	1:80	Neg.	+
6. H. L.†	55F	Felty's	4 cm.	2.2		1:320	Neg.	+
7. H. C.	47F	Felty's	10 cm.	0.8	2.8/3.0	1:5120	Neg.	+
			(1958)‡	6.6§	2.6/1.9§	1:2560§	Neg.§	+§
8. L. M.	58F	Felty's	3 cm.	1.6	3.6/4.8			
			(1953)‡	6.6§	3.7/3.7§	1:640§	Neg.§	+§
9. T. Mc.†	65M	Felty's	5 cm.	1.7	3.9/3.9		Neg.	
			(1958)‡	8.9§		1:160§	Neg.§	+§
10. L. Mc.	61F	Felty's	3 cm.	1.4	5.1/3.3			
			(1953)‡	5.6§	4.5/3.1§	1:40§	Neg.§	+§
1. L. B.	54F	Rh. Arth.	Not Palp.	5.0	3.7/3.0	1:320	Neg.	+
2. H. W.	33M	Rh. Arth.	Not Palp.	6.8	4.7/3.4	1:1280	Neg.	+
3. C. V.	35M	Rh. Arth.	Not Palp.	10.0	4.3/2.1	1:2560	Neg.	Neg.
4. C. D.	54M	Rh. Arth.	Not Palp.	7.8	4.1/2.6	1:1500	Neg.	Neg.
5. A. J.	71M	Rh. Arth.	Not Palp.	8.5	4.0/3.6	1:160	Neg.	Neg.
6. L. S.	27F	Rh. Arth.	Not Palp.	8.5	3.9/2.3	1:40	Neg.	Neg.
7. M. J.	57F	Rh. Arth.	Not Palp.	6.5	2.6/3.0	1:320	Neg.	Neg.
8. W. M.	60M	Rh. Arth.	Not Palp.	7.4	4.7/2.2	1:20		Neg.
9. C. Mc.	55M	Rh. Arth.	Not Palp.	7.8	4.2/2.4		Neg.	Neg.
10. H. S.	50F	Rh. Arth.	Not Palp.	8.5	4.9/2.2	1:320	Neg.	Neg.
11. M. D.	56F	Rh. Arth.	Not Palp.	12.0		1:20	Neg.	Neg.
12. H. B.	66M	Rh. Arth.	Not Palp.	11.5		Neg.	Neg.	Neg.
13. G. H.	65M	Rh. Arth.	Not Palp.	6.3	4.1/2.6	1:1280	Neg.	Neg.

* Negative at time of positive fluorescent antiglobulin test. Two months later positive L.E. preps.
† We are grateful to Dr. W. C. Moloney, Boston, Mass. for allowing us to study this patient.
‡ Date of splenectomy.
§ Postsplenectomy determinations.
Blank spaces, no determination performed.

dermatitis. Hemolytic anemia and thrombocytopenia were absent and the serological test for syphilis (VDRL) was negative. She had received infrequent small doses of steroids intermittently since 1954.

The sera from each of these 10 patients produced strong nuclear fluorescence which resembled in all respects the response obtained with the sera from the patients with SLE (Figures 1*a* and 1*c*). The presence of positive nuclear fluorescence tests in four patients (L.M., L.Mc., T.Mc. and H.C.) for periods up to five years after splenectomy, in the presence of normal leukocyte counts, is of particular interest.

Of the remaining 13 patients with uncomplicated rheumatoid arthritis, the sera from two

contained a factor which caused nuclear fluorescence. None of these patients had positive L.E. cell tests.

A group of eight patients with other forms of possibly related disorders, all without splenomegaly or leukopenia and with negative L.E. cell tests, showed negative results with the fluorescent staining technique (Table I).

Nonrheumatic patients with hyperglobulinemia or on steroid therapy

To test the possibility that hyperglobulinemia *per se* may produce a positive reaction, the sera of seven patients with elevated globulins were tested. Three of these had Laennec's cirrhosis with globulins ranging from 3.0 to 3.8 Gm. per 100 ml. Three had multiple myeloma with globulins of 5.9, 11.2 and 11.3 Gm. per 100 ml., respectively, and one patient with bronchogenic carcinoma had a globulin of 4.6 Gm. per 100 ml. The fluorescent antiglobulin test was negative in all.

To exclude a possible effect of steroid therapy on the mechanism of the test, the sera of three patients with chronic bronchial asthma were tested. All had been receiving significant doses of steroids for at least three years and all showed negative results.

Patients receiving multiple transfusions

The sera from four patients who had received multiple transfusions were studied. Three of these patients had received 80, 97 and 232 transfusions, respectively, and two of them experienced repeated, nonhemolytic, febrile reactions. The serum from the fourth patient was known to contain a powerful leukoagglutinin.[2] A noticeable increase of leukocyte fluorescence was observed in all. The location of this was at first interpreted to be nuclear (31), but with the greater magnification and better resolution of the microscopic equipment now available to us, it is clear that all the fluorescence is confined to the cytoplasm.

Idiopathic thrombocytopenic purpura

Because it is known that destructive processes of the three formed elements of the peripheral blood may on occasions show multiple involvement (1, 4, 13, 40), sera from five patients with idiopathic thrombocytopenic purpura were studied for evidence of antileukocyte globulins. Leukocyte fluorescence was negative in all cases, but particularly striking platelet fluorescence was observed in one case. This suggests the possibility of developing a similar fluorescent antiglobulin test for platelets.

Leukemias, polycythemia vera and Hodgkin's disease

A total of 27 patients with these disorders were studied, including six cases with aleukemic leukemia (Table I). In none was the presence of antileukocyte factors demonstrated by the present technique.

In vivo studies

Three hundred fifty ml. of I.B.'s plasma and 250 ml. of R.S.'s plasma, both with clinically diagnosed Felty's syndrome, were administered to two hematologically normal recipients. Within 15 minutes from the beginning of the infusion a significant fall of the total leukocyte count was observed in each recipient (Figure 2*a*). There was no selective depression of either granulocytes or mononuclear cells. In one case (plasma from I.B.), the transfusion was followed by shaking chills for 45 minutes, accompanied by an urge to urinate, and in both instances the recipients experienced a transient temperature elevation to 100° and 101° F., respectively (Figure 2*a*). Identical infusions of normal plasma administered two days following each experiment produced no systemic reaction or significant change in leukocyte count in either recipient (Figure 2*b*). The infusion of 250 ml. of plasma from A.T. (SLE) into a control recipient produced no significant change in the leukocyte count.

DISCUSSION

The study of leukocyte immunology in clinical investigations has been based chiefly on the *in vitro* demonstration of leukoagglutinins and *in vivo* transfusion or transplacental (41) transfer of antileukocyte factors. The problems and inaccuracies of the first method stem mainly from the difficulty in preparing adequate leukocyte

[2] We are grateful to Dr. T. E. Brittingham, St. Louis, Mo., for sending us this serum.

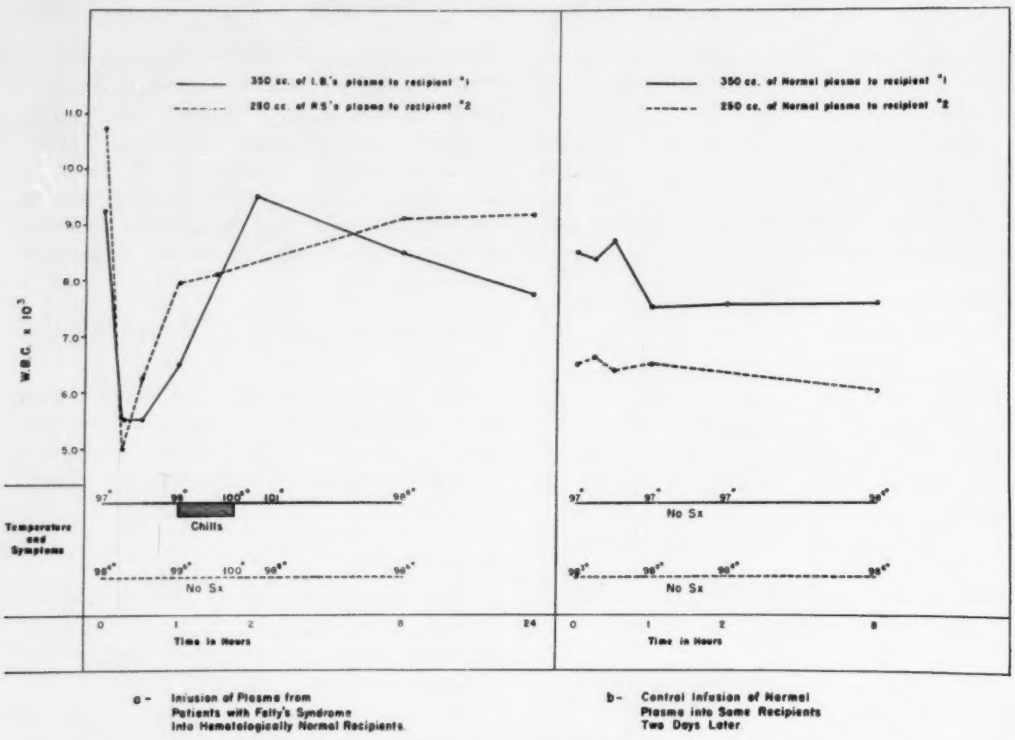

FIG. 2. RESULTS OF PLASMA INFUSIONS

In all cases the infusions were begun at zero time. They were terminated at 30 minutes in recipient No. 1 and at 60 minutes in recipient No. 2.

suspensions without causing injury and hence alteration of the cell surface, which results in false positive agglutination (6). In addition, agglutinations may occur from fibrinogen adsorbed on the surface of normal leukocytes (42), from leukergy (43), as well as from red and white cell clumps either secondary to erythrophagocytosis (28, 35) or nonspecific cellular adherence (44). The hazards (23) and inherent limitations of the *in vivo* studies preclude their extensive use in clinical practice. Neither technique is readily applicable to the study of autoimmune leukopenia or to the demonstration of incomplete and cell-bound leukocyte antibodies (25). Finally, it is difficult with either method to differentiate between immunologic factors directed against the various types of leukocytes, and both fail to detect specifically antinuclear and anticytoplasmic factors. The fluorescent antiglobulin technique is theoretically capable of circumventing these difficulties. The use of this technique for the study of leukocyte immunology has been hampered by the fact that nonspecific fluorescent staining of the leukocyte cystoplasm occurs with labeled rabbit anti-human globulin (Coombs') serum (29, 33, 34, 45). The precise mechanism of this phenomenon is not clear, but it may be due to nonspecific adsorption of plasma proteins on the hydrophilic polar surface of the leukocytes (42). In our hands, nonspecific fluorescence occurring with untreated labeled antiglobulin made the differentiation of positive from negative preparations very difficult and often impossible. However, absorption of the labeled conjugate with acetone-dried rabbit bone marrow before use (33, 34) greatly diminishes or entirely eliminates the nonspecific cytoplasmic fluorescence (compare Figures 1*b* and 1*d*). This step greatly facilitates the detection of specific antinuclear reactions by providing definite contrast between nuclear fluorescence and cyto-

plasmic background (Figures 1*a* and 1*c*). Furthermore it may prove valuable in studying specific anticytoplasmic substances which could not be otherwise accurately quantified in the presence of this coexisting nonspecific fluorescence. Although an increased cytoplasmic localization was present when absorbed antiglobulin was superimposed on the sera of patients receiving multiple transfusions, it is evident that further carefully controlled studies are required to ascertain whether this observed difference is reproducible.

Previous animal experiments (46, 47) have indicated that two types of heteroimmune granulocyte antibodies may be produced. One of these is directed against the cytoplasm and may be obtained by injecting intact cells (47), whole cytoplasm (46, 48) or cytoplasmic fractions (46, 49). The antiserum thus produced appears to promote ingestion of the intact granulocyte with lytic effects upon the cytoplasm (46, 47) and is said to be specific for leukocytes (46). The other type of antibody may be produced by injecting nuclear material from granulocytes (48) or other tissues and hence appears to be relatively nonspecific (46). Although administration of this antiserum to animals produces a much less pronounced granulocytopenia than anticytoplasmic serum (48), when combined with normal granulocytes it is capable of producing "L.E.-like" cells (46). It has been recently reported by Friou, Finch and Detre (50) and Holman and Kunkel (51) that the fluorescent antiglobulin technique could be employed to demonstrate the interaction of the serum L.E. factor and cell nuclei and nucleoprotein. Moreover, it appears that nuclear material from many different species and varying human tissues can be made to participate in this reaction (45, 50–53). Our results have confirmed the work reported by others indicating the affinity of the serum of patients with SLE for leukocyte nuclei. Furthermore, in one instance, this test has proven more sensitive than the conventional L.E. cell preparation, anticipating the demonstration of the latter by at least two months. A similar case, with detection of "lupus globulin" preceding the L.E. cell test by two years, has recently been reported in detail by Friou (54).

Since 1924, when Felty (55) first described a syndrome consisting of leukopenia, splenomegaly and arthritis, there has been much speculation regarding the nature of this entity and the cause of the leukopenia. It is generally accepted that this represents a variant of rheumatoid arthritis (56–58). The leukopenia, often a neutropenia, has been variously attributed to chronic infection (57), hypersplenism (56, 58–60), leukocyte agglutinins (17) or unknown causes (61). It is well recognized that a positive L.E. cell test can be obtained in rheumatoid arthritis (54, 62–64), particularly in Felty's syndrome (65). In addition, the presence of small quantities of "lupus globulin" has been detected in patients with rheumatoid arthritis (54). The finding of positive nuclear fluorescence in 12 of our cases of rheumatoid arthritis is therefore not entirely unexpected. It is particularly noteworthy, however, that all 10 cases of Felty's syndrome, without exception, showed a strongly positive reaction indistinguishable, by our method, from that seen with SLE. To our knowledge this has not been previously reported. This finding offers further evidence of a basic relationship in these disease processes and encourages speculation that we may be dealing with a similar pathologic entity with a wide spectrum of manifestations. Thus, Felty's syndrome may be a connecting link which lies somewhere between uncomplicated rheumatoid arthritis and SLE.

Some authors have considered that the L.E. factor may be responsible for the development of the leukopenia commonly seen in SLE (46, 66). We would like to suggest that an antinuclear globulin may also be partly responsible for the leukopenia seen in Felty's syndrome. Further evidence that an antileukocyte factor in Felty's syndrome may be involved in the pathogenesis of the leukopenia was obtained from *in vivo* experiments. Definite, immediate, transient depressions of the leukocyte count accompanied by chills and fever, were obtained upon transfusion of 250 and 350 ml. of sera containing this factor. The quality and duration of this response are similar to those described by others (4, 6, 17, 67–69). Presumably the fever was secondary to the release of tissue pyrogens from destroyed leukocytes (6, 70, 71); however, notwithstanding sterile cultures, the possible presence of bacterial pyrogens cannot be entirely excluded (72).

Recent work has demonstrated that patients who have received multiple transfusions may de-

velop leukoagglutinins (23, 68, 73) or incomplete white cell antibodies (74) in their sera. Moreover, isoimmunization of a normal subject has been achieved by repeated injection of leukemic blood (23). The practical importance of this phenomenon in producing febrile transfusion reactions has been stressed, and their prevention by removal of leukocytes prior to transfusion is advocated (22, 68, 75). If an inference can be drawn from the previously discussed animal experiments (46–49), it would seem reasonable to find that these presumed human leukocyte isoantibodies are primarily directed against cytoplasmic antigens.

There are many other conditions in which antileukocyte substances have been demonstrated or suspected. The application of the fluorescent antiglobulin technique for the study of sera from such patients should be of help in elucidating the mechanisms of the leukopenia, the type of cells involved, the character of the substances responsible and their location on the cell.

SUMMARY

A method for studying leukocyte immunology by the fluorescent antiglobulin technique is described.

The results with sera from various disease states are reported. The data presented are consistent with the concept that human antileukocyte globulins may be directed against the nucleus or against the cytoplasm.

Antinuclear globulins were detected in the sera of all patients with systemic lupus erythematosus (SLE) and Felty's syndrome studied, and in two cases of apparently uncomplicated rheumatoid arthritis. These findings suggest that Felty's syndrome may be a connecting link of a disease spectrum involving simple rheumatoid arthritis and SLE.

Evidence that a circulating factor present in patients with Felty's syndrome may be involved in the pathogenesis of the leukopenia was obtained in two instances by plasma transfusions.

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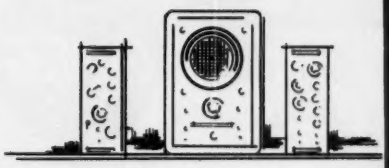

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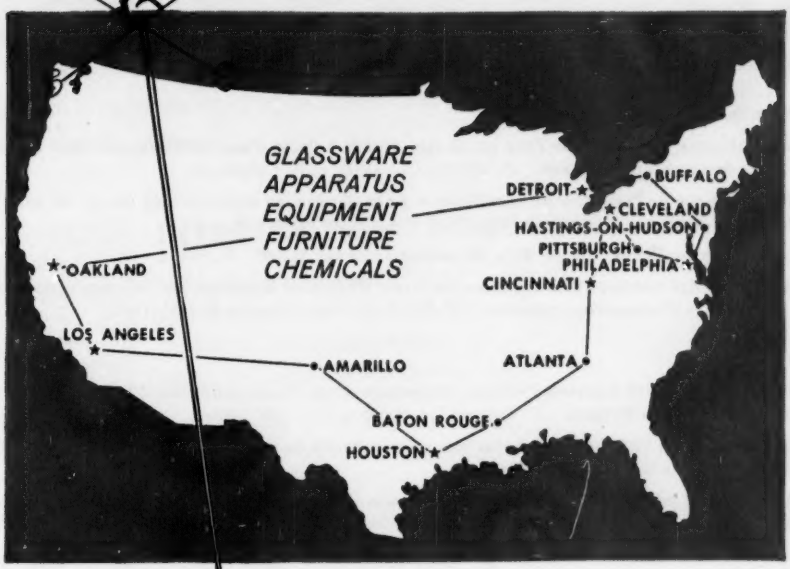

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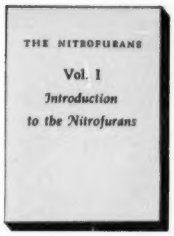

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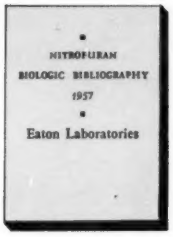

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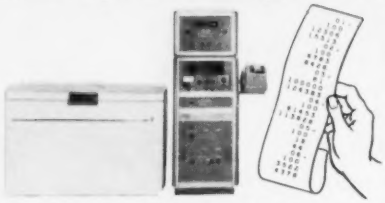

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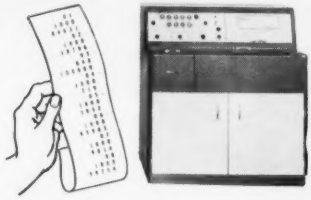

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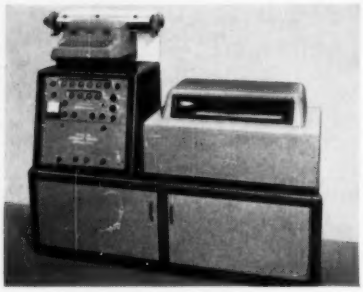

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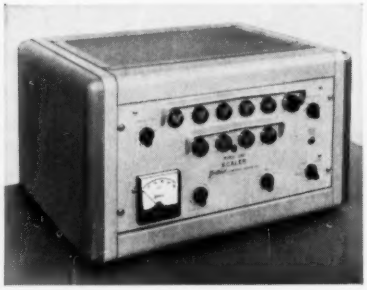

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